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13. ABSTRACT *(Maximum 200 Words)* Vitamin D or 1,25-dihydroxycholecalciferol (calcitriol)inhibits proliferation, induces differentiation and modulates cell cycle progression in a variety of normal and malignant cells. We demonstrated in the Dunning rate prostate cancer(Mat-Ly-Lu) and a human xenograft model (PC-3) that calcitriol has significant anti-proliferative activity, arrests cells in GO/G1, modulates expression of p27 and p21, induces PARP cleavage and significantly enhances the anti-tumor activity of conventional chemotherapeutic agents, especially paclitaxel. In addition, we have completed a phase I trial of high dose calcitriol in which we determined the MTD of subcutaneous calcitriol and calcitriol pharmacokinetics. Since paclitaxel has considerable potential as an agent for the therapy of prostate cancer and calcitriol potentiates paclitaxel and is active as a single agent, we propose to examine further the therapeutic potential of calcitriol by: 1) determining the schedule and time-dependent parameters of calcitriol and paclitaxel for optimum potentiating activity in MLL and PC-3 prostate cancer models; 2) determining the role of calcitriol in the induction of apoptosis and the role of changes in intracellular Ca+2 in these activities; and 3) evaluating the toxicities, MTD, pharmacokinetics and pharmacodynamics and activity of paclitaxel and calcitriol when administered to patients with prostate cancer.

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Introduction

In addition to its classical role in bone and mineral metabolism, vitamin D (calcitriol, 1,25-dihydroxycholecalciferol) has antiproliferative activity in solid tumor models both in vitro (1-7) and in vivo (1, 4, 7, 8). Calcitriol binds to the vitamin D receptor (VDR), a member of the steroid nuclear receptor superfamily, which results in the transcription of target genes (9). We demonstrated that calcitriol inhibits the growth of the murine squamous cell carcinoma (SCC) (7) and the Dunning rat prostatic adenocarcinoma, Mat-lylu (1). In addition, calcitriol in combination with cisplatin or carboplatin synergistically enhanced anti-tumor activity of in vitro and in vivo, and these effects were schedule dependent (13). Mechanistic studies reveal that calcitriol induces G_0/G_1 arrest, a decrease in Rb phosphorylation, an increase in expression of p27, and a decrease in expression of p21, cyclin dependent kinase inhibitors (10-14). Recent studies indicate a reduction in p21 expression sensitizes tumor cells to both DNA damaging agents (15,16), and microtubule damaging agents such as paclitaxel (17, 18, 19). Given the ability of calcitriol to decrease p21 expression in vitro and in vivo, and the reported association between reduced expression of p21 and increased sensitivity to paclitaxel, we hypothesized that calcitriol would enhance the antitumor activity and apoptosis-promoting ability of paclitaxel. Studies also have investigated the mechanism by which calcitriol induces apoptosis, particularly with respect to its effects on survival and stress signaling pathways. Calcitriol induces the caspase-dependent cleavage of MEK, a target of proteolytic degradation not previously described, resulting in nearly complete loss of MEK expression and Erk1/2 signaling. Direct comparison of calcitriol with genotoxic agents revealed that the molecular events described above, with the exception of Akt inhibition, were selective for calcitriol. We present here our results to investigate calcitriol-mediated tumor cell death and the interactions of paclitaxel and

calcitriol both pre-clinically and clinically with the support of Department of Defense grant DAMD17-98-1-8549. We have chosen to evaluate combinations of calcitriol with and without paclitaxel in PC-3 (a human prostate cancer) and SCC (a murine squamous cell carcinoma) to investigate differences in response and biologic effects, hypothesizing that such differences may provide insight into the mechanism of calcitriol-mediated cell death in prostate cancer and in the interaction between these two agents. In addition, we present data on the ongoing clinical trial involving calcitriol and paclitaxel with plans for a trial involving calcitriol/dexamethasone in combination with docetaxel and zolendronic acid in men with hormone refractory prostate cancer.

Body:

Statement of Work- Specific Aim 1: To determine the parameters for optimum potentiating activity in prostate model systems

Calcitriol increases paclitaxel anti-tumor activity in vitro. To examine the antitumor activity of calcitriol and paclitaxel alone, or in combination, murine SCC cells were used in an in vitro clonogenic assay. SCC cells were (a) treated with paclitaxel or calcitriol alone, (b) were pre-treated for 24h with calcitriol then treated with paclitaxel, or (c) were treated simultaneously with both agents. As we previously reported, calcitriol inhibits clonogenic survival in SCC with an IC50 of 4 nM (Fig. 1 and (7)). Paclitaxel alone also inhibits SCC survival with an IC50 of 23 nM. Significantly greater antitumor activity was achieved when calcitriol was combined with paclitaxel (Fig. 1). Calcitriol pre-treatment potentiated paclitaxel activity to a greater extent than concurrent treatment, except at the highest dose of paclitaxel studied. These results demonstrate that antitumor activity in SCC is increased by combining calcitriol with paclitaxel, and that the optimal schedule for administration is treatment with calcitriol followed by paclitaxel.

Similarly, we examined whether the combination of calcitriol and paclitaxel was effective in inhibiting the growth of human prostatic adenocarcinoma cells (PC-3). PC-3 cells were treated in vitro for 24h with or without calcitriol and received no further treatment or were treated for an additional 24h with varying concentrations of paclitaxel. As shown in Fig. 2, calcitriol alone had detectable antiproliferative activity in these cells, with an IC50 of 5 μM. Paclitaxel, when used as a single agent, reduced PC-3 clonogenic survival in a concentration-dependent manner. Significantly greater growth inhibition was achieved by pre-treating the cells with calcitriol at

each of the paclitaxel concentrations tested. Calcitriol also enhanced paclitaxel-mediated growth inhibition in vitro in the Dunning rat metastatic adenocarcinoma (MLL) model system (data not shown). Therefore, calcitriol increases paclitaxel activity in murine squamous cell carcinoma and human as well as rat prostatic adenocarcinoma. This indicates the effects are not cell-type specific.

Calcitriol increases paclitaxel anti-tumor activity in vivo. To evaluate whether the combination of calcitriol and paclitaxel has greater in vivo antitumor activity compared to either agent alone, the excision clonogenic assay was used. As shown in Fig. 3A, the combination of calcitriol plus paclitaxel resulted in a significantly greater decrease in surviving fraction as compared to paclitaxel or calcitriol alone. Thus, greater in vivo antitumor activity is achieved in SCC by pre-treatment with calcitriol followed by paclitaxel.

To determine whether an increase in clonogenic cell kill was associated with inhibition of tumor growth, SCC tumor-bearing mice were treated with saline, calcitriol or paclitaxel alone, or calcitriol in combination with paclitaxel. We utilized a schedule of daily × 3 doses of calcitriol with paclitaxel administered on day 3. This calcitriol dosing regimen was previously reported to maximize antitumor efficacy while minimizing toxicity or hypercalcemia (13). In SCC, paclitaxel had no significant activity when used as a single agent therapy and calcitriol alone exhibited cytostatic activity (Fig. 3B). In contrast, the combination of calcitriol with paclitaxel resulted in significant tumor regression (Fig. 3B).

To determine whether calcitriol plus paclitaxel combination therapy also displays increased antiproliferative activity in vivo in PC-3, tumor-bearing mice were treated with saline, calcitriol or paclitaxel alone, or calcitriol in combination with paclitaxel utilizing the dosing schedule described for SCC. As shown in Fig. 4A, neither paclitaxel nor calcitriol had significant activity when used as a single agent therapy in PC-3. However, significant antitumor activity was observed when PC-3 tumor-bearing mice were treated with calcitriol followed by paclitaxel (Fig. 4A). In this model, nearly complete inhibition of tumor growth was maintained for greater than two weeks.

After day 24, PC-3 tumors in animals treated with calcitriol plus paclitaxel achieved a growth rate comparable to controls as determined by the slope of the growth curve (0.107 vs. 0.102). Re-treatment of these animals with calcitriol plus paclitaxel on days 29-31 resulted in a decrease in the rate of tumor growth, as evidenced by a change in the slope of the growth curve for days 32-38 (m= 0.052) (Fig. 4B). These data indicate that previously treated tumors remain responsive to calcitriol plus paclitaxel and that prolonged antitumor activity may be achieved by repeated cycles of therapy.

Statement of Work- Specific Aim 2: To determine the role of calcitriol in the induction of apoptosis and in changes in calcium

Paclitaxel effects on calcitriol-induced hypercalcemia. We have examined the effect of calcitriol and paclitaxel on tumor regression in nude mice bearing PC-3 xenografts (calcitriol 1.25μg/day x3d + paclitaxel 20mg/kg, d3). The combination of calcitriol plus paclitaxel resulted in marked delay in tumor growth. We have demonstrated identical activity of this combination in the syngeneic Dunning rat prostate model, MLL. Treatment with paclitaxel or calcitriol alone resulted in limited anti-tumor effect in both systems. In addition to the enhancement of the

antitumor effect of paclitaxel by calcitriol we also noted that the paclitaxel + calcitriol treated animals had less hypercalcemia than animals receiving calcitriol alone. (Table 1) Paclitaxel reduces urinary calcium excretion suggesting that any protective effect is likely related to effects of paclitaxel on calcitriol-mediated actions on gastrointestinal calcium absorption or bone mobilization of calcium stores. Agents such as paclitaxel that disrupt microtubules cause parathyroid hormone dependent changes in Ca2+ transport and thereby abrogate hypercalcemia (36). Studies exploring the mechanisms and clinical application of these interactions are underway.

Table 1

Serum and Urinary Calcium in C3H/HEJ Mice Following Calcitriol, Paclitaxel and Zoledronic Acid Treatment

	Day 3E	Day 4F	24 hr Urinary CalciumG
Control	9.5 + /-0.2	10.2 +/- 0.4	7.5 +/- 0.1
DDDA	16.9+/-0.7	18.8+/-1.4	40.2 +/- 0.8
DDDB T	9.9+/-1.5	12.2 +/- 1.3	6.4 +/- 0.3
Tc	9.6+/-0.2	9.8+/-0.3	N.D.
Zoledronic acid + DDDH	12.8+/-0.3	13.2+/-0.2	N.D.
Zoledronic acid	9.5+/-0.5	9.3+/-0.2	N.D.

A=calcitriol 0.75μg/kg/d, Day 1,2,3; B=calcitriol 0.75μg/kg/d, Day 1,2,3 + paclitaxel (20mg/; C = paclitaxel (20mg/kg). E = calcium measured after day 3 of calcitriol administration; F = calcium measured one day after last calcitriol administration; G= 24 hour urine calcium measured over the last 24 hours of therapy. H= Zoledronic acid 10mg/kg day –1, calcitriol day 1,2 and 3.

We have developed preclinical data in the previously noted animal model that docetaxel blocks calcitriol induced hypercalcemia at least as effectively as paclitaxel – and calcitriol potentiates docetaxel antitumor effects in PC-3 in vivo. In addition, the bisphosphonate, zoledronic acid, also effectively blocks calcitriol-induced hypercalcemia. Fig 5 illustrates the effects of calcitriol, dexamethasone (dex) and docetaxel therapy in nude mice bearing human

prostate cancer (PC-3) xenografts. We have shown previously that dexamethasone (dex) alone has little effect on tumor cell growth.

Calcitriol accelerates paclitaxel-induced apoptosis independent of its effects on p21. Given the findings that loss of p21 sensitizes MCF-7 and HCT116 cells to paclitaxel (17, 18), and calcitriol decreases p21 expression in SCC (14), we hypothesized that calcitriol enhances paclitaxel antitumor activity via its effects on p21. To test whether calcitriol treatment decreases p21 expression in PC-3 as it does in SCC, cells were treated in vitro with EtOH vehicle control or calcitriol. At various times, whole cell lysates were prepared and analyzed for p21 expression by Western blot. Calcitriol treatment resulted in a 60% decrease in p21 expression in PC-3 cells after 72h and an 80% decrease in expression after 96h (Fig. 6).

Paclitaxel-induced apoptosis in PC-3 cells is associated with phosphorylation of Bcl-2 (20), a modification that inactivates the apoptotic suppressor function of this protein (20-22). To determine whether PC-3 cells with reduced p21 expression show enhanced paclitaxel activity, we examined whether calcitriol pretreatment increased or accelerated paclitaxel-induced changes in Bcl-2 expression and apoptosis. PC-3 cells were either untreated or pre-treated with 5 μM calcitriol for 72h, a time sufficient for p21 down-modulation. Subsequently, cells either received no further treatment or were treated with paclitaxel for varying lengths of time. As a further control, an additional set of cells was treated concurrently with calcitriol plus paclitaxel; under these conditions, the calcitriol-mediated reduction in p21 would not occur prior to paclitaxel exposure. Inspection of treated cells revealed two morphologically distinct populations; one cell

population remained adherent following treatment while a second population detached from the culture dishes (floating cells). These populations were analyzed separately.

At 24h, paclitaxel induced Bcl-2 phosphorylation in both the adherent and detached cell populations (Fig. 7A). Whereas unphosphorylated Bcl-2 was most abundant in the paclitaxel-treated adherent cells, only the phosphorylated forms of Bcl-2 were detected in the detached cells. Phosphorylated Bcl-2 species were still detected in both cell populations after 48h of treatment, but were virtually absent by 72h. Calcitriol had little effect on Bcl-2, and neither pre-treatment nor concurrent treatment with calcitriol altered the effects of paclitaxel on Bcl-2 expression/phosphorylation.

Apoptosis, (as measured by the cleavage of the caspase substrate poly(ADP-ribose) polymerase, or PARP), first became evident in the detached cells after 48h of treatment (Fig. 7B). At this time, paclitaxel treatment resulted in a 50% reduction in PARP. Although calcitriol itself did not induce apoptosis, it enhanced the effects of paclitaxel such that cells treated with calcitriol prior to or in combination with paclitaxel displayed a 78% reduction in PARP. By 72h, PARP was no longer detected in the cells that detached following paclitaxel administration, and addition of calcitriol had no further discernible effect. Calcitriol did not alter the effects of paclitaxel on PARP expression in the adherent cell population at any of the times examined. The data obtained at 48h indicate calcitriol can accelerate paclitaxel-induced apoptosis in a subset of PC-3 cells.

Calcitriol induces caspase-dependent cleavage of MEK in apoptotic cells. We assessed induction of apoptosis by calcitriol on the molecular level by examining PARP in attached and detached (floating) tumor cell populations. After treating tumor cells (SCC and PC-3) as described above, the floating cells were removed from the attached cells and lysates of both cell populations were prepared. The number of floating cells in the medium after vehicle treatment was low and it was not possible to collect sufficient amounts to process for immunoblot analysis. As shown in Fig. 8, lysates of vehicle-treated (attached) cells, as well as the attached cell population of calcitriol-treated cultures, exhibited no PARP cleavage. However, lysates of floating cells from cultures treated with calcitriol demonstrated complete PARP cleavage. Taken together, these results indicate that calcitriol induces programmed cell death in tumor cells. The fact that PARP cleavage was exclusively observed in lysates from calcitriol-treated, non-attached cells and that this population is easily isolated from the attached cell population affords the use of this model for investigating the role of signaling pathways in calcitriol-induced apoptosis at the molecular level.

The Ras-Raf1-MEK-Erk signaling pathway transduces signals from growth factor receptors to the nucleus which can lead not only to mitogenesis and differentiation but also to survival (23). Thus, growth factor signaling may attenuate the apoptotic signal induced by calcitriol, or, conversely, calcitriol may attenuate growth factor signaling as part of its mechanism to promote programmed cell death. We investigated the effects of calcitriol on levels of activated MEK, a key mediator of signaling through the Ras-Raf1-MEK-Erk pathway. Levels of phosphorylated/activated Erk1/2 were analyzed and found to be virtually undetectable in the lysates of the floating cells (Fig. 9), indicating that the Ras-Raf1-MEK-Erk signaling pathway is completely blocked in these cells. It is perhaps noteworthy that calcitriol also modestly inhibited Erk1/2 in calcitriol-treated attached cells despite the fact that levels of

phosphorylated MEK and intact MEK protein were unaffected. The molecular basis of calcitriol-induced Erk1/2 inhibition in attached cells is currently under investigation. Erk1/2 expression was unaltered in lysates of calcitriol-treated attached cells, and, in contrast to MEK, was only slightly reduced in floating cells.

Since it is well established that programmed cell death often involves the activation of caspases, we examined whether these proteases might be responsible for calcitriol-induced MEK proteolysis. SCC cells were treated 2 days either with vehicle, calcitriol alone, or calcitriol in the presence of either DEVD-FMK or zVAD-FMK. While the caspase-3 inhibitor DEVD-FMK had little effect on calcitriol-induced MEK proteolysis, the pan-caspase inhibitor zVAD-FMK nearly completely blocked the loss of MEK protein and the production of the 33 kDa MEK fragment (Fig. 10). Thus, the decrease in MEK expression induced by calcitriol treatment does not appear to be due to inhibition of MEK protein synthesis, but rather the result of caspase-dependent (not necessarily caspase-mediated) MEK cleavage. Caspase-dependent cleavage of MEK has not been previously reported. It is perhaps noteworthy that the apparent sum of the levels of intact MEK and the 33 kDa fragment in lysates of cells treated with calcitriol alone appears to be less than the total level of MEK in cells co-treated with calcitriol and zVAD-FMK.

Calcitriol inhibits the Akt survival signaling pathway. In addition to the MEK-Erk pathway, a significant survival signal is also generated by the PI-3-kinase-Akt pathway. Akt was recently demonstrated to undergo caspase-dependent cleavage during apoptosis induced by treatment either with etoposide, ultraviolet-C exposure, or Fas ligation in human Jurkat leukemia cells (24). To assess whether this pathway is also affected in calcitriol-induced apoptosis,

immunoblot analysis was used to assess Akt phosphorylation and expression in lysates of cells treated with either vehicle or calcitriol. Fig 11 shows that calcitriol inhibited Akt in a manner similar to that of MEK effecting a strong decrease in both its phosphorylation and expression.

Calcitriol induces MEKK-1 expression in apoptotic and non-apoptotic cells and promotes MEKK-1 proteolysis in apoptotic cells. Although the above results clearly indicate that cells undergoing vitamin calcitriol-induced apoptosis exhibit a block in the mitogenic/survival signaling pathways, this alone may not be sufficient to induce cells to enter programmed cell death. Therefore, in an effort to assess whether calcitriol treatment also activates stress signals that could directly promote apoptosis, tunor cells were treated with or without calcitriol and the expression of MEKK-1, an upstream activator of the SEK1-JNK and the MKK3/MKK6-p38 stress pathways, assessed by Western blot analysis. As shown in Fig. 12, calcitriol significantly up-regulated MEKK-1 expression in both the attached and floating cell populations, with expression being greater in the floating, apoptotic cells. Longer exposures of the X-ray film to the Western blot ECL signal revealed multiple minor immuno-reactive bands in the lysates of floating cells (MW range of 85 – 190 kDa) that were not observed in those of attached cell. Since the anti-MEKK-1 antibody recognizes an epitope lying very near the C-terminus of the MEKK-1 protein, these data indicate that the products formed represent MEKK-1 species that have undergone N-terminal proteolysis. Thus, some or all of the lower molecular weight anti-MEKK-1 positive bands produced upon treatment with calcitriol may represent MEKK-1 proteolytic products displaying constitutive kinase activity.

MEK cleavage and MEKK-1 up-regulation are not significantly induced by cisplatin. We next addressed whether the effects of calcitriol on MEK and MEKK-1 described above were general phenomena of tumor cells that could be observed during apoptosis induced by other agents or if they were selectively induced by treatment with calcitriol. Cells were treated either with calcitriol (10 nM) or with cisplatin (cDDP, 1 ug/ml) for 2 days, the attached and non-attached cell populations were separated, and lysates were prepared of both. As shown in Fig. 13, calcitriol and cisplatin both induced PARP cleavage in t□e floating cell populations, but not in the attached cell populations, demonstrating that the floating cells from both treatments represent only those cells induced to undergo apoptosis. Significant loss of MEK expression and MEK cleavage, however, were only observed for lysates of apoptotic cells from calcitriol–treated cultures. Although the 33 kDa MEK fragment could be observed in lysates of cisplatin-treated floating cells, it was typically observed at levels less than those observed for lysates of calcitriol–treated floating cells. In addition, significant up-regulation of MEKK-1 was observed only for calcitriol–treated cells, particularly in the non-attached apoptotic population, and limited N-terminal MEKK-1 proteolysis was exclusively observed in the calcitriol–treated, detached cells. Although a slight induction of MEKK-1 was observed for cisplatin-treated attached cells, this induction was no longer observed in the floating cells. Thus, caspase-dependent MEK cleavage and MEKK-1 up-regulation/proteolysis are not general pheneomena of apoptosis observed in these cells, but are selectively induced by calcitriol. Taken together, these results suggest that calcitriol induces apoptosis in tumor cells by a mechanism that is distinct from that of cytotoxic agents.

Statement of Work- Specific Aim 3: To evaluate the toxicities, MTD, pharmacokinetics of paclitaxel and calcitriol in patients with advanced prostate cancer

Clinical trial results to date.

We have treated 30 patients on the clinical trial proposed in this grant. The following is the treatment schedule utilized:

Day	1	2	3	4	5	6	7	8	9	10
Calcitriol	*	*	*					*	*	*
Paclitaxel	80mg									80mg
PK	♦									♦

Paclitaxel is given at a dose of 80mg/sq m D1, D10, D17 and D23, every 6 weeks

Calcitriol is administered weekly by mouth according to the following dose escalation scheme: 4, 6, 8, 11, 13, 17, 22, 29, 38 and at 30% increments for subsequent escalation in cohorts of 3-6 patients. Studies were designed such that in each patient, paclitaxel was given on day 1 before calcitriol in one treatment and on day 10 after 3 days of high dose calcitriol in the next cycle. This permits comparison of AUC of either agent in the same patient before and after pretreatment with calcitriol. We have entered 30 patients on trial and have completed escalations through 38 μg p.o. QDX3 weekly + paclitaxel. Dose limiting toxicity has not been encountered; no patient has experienced a serum calcium (corrected) >11mg/dL. No significant myelosuppression was seen where ANC was a reflection of the cycles of paclitaxel with no difference observed in the presence of calcitriol (Fig14). Blood samples to assess plasma pharmacokinetics of paclitaxel when administered on Day 1 with the initiation of calcitriol and

on Day 10, after 3 days of calcitriol have been collected, as have samples for assessment of calcitriol pharmacokinetics. As shown in Fig 15, the AUC of paclitaxel was not different with or without pretreatment with calcitriol from a representative patient and when compared over the doses of calcitriol tested to date (Fig 16). No difference in toxicity between week 1 and week 2 of paclitaxel as been noted, suggesting that there is no reduction in paclitaxel clearance associated with calcitriol administration. Calcitriol pharmacokinetic study determined that as dose increased, AUC increased until we reached dose levels of calcitriol of greater than 17μg (Fig 17) where it appears that we have reached saturation. Additionally, none of these patients were hypercalcemic. Studies are ongoing to examine the metabolites of calcitriol in these patient samples and whether we indeed have a bioavailablity issue and can not escalate further.

We are also conducting a concurrent phase 2 trial in androgen-independent prostate cancer of calcitriol (Monday, Tuesday and Wednesday) + dexamethasone (4mg Sunday, Monday, Tuesday and Wednesday). In this study we have treated 24 patients with 12μg QDX3 of calcitriol weekly (total weekly dose 38μg/week) for at least one month. No patient has had hypercalcemia. Our prior studies of calcitriol alone administered on a daily oral basis indicate that at least 30% of individuals will develop hypercalcemia at a weekly dose of 7.5-14μg/ week. In individuals treated with calcitriol to retard the development of osteoporosis, doses of calcitriol of >14μg/week are frequently associated with hypercalcemia. Hence, failure to observe hypercalcemia in the present paclitaxel + calcitriol trial despite administering a weekly dose of calcitriol substantially higher than one would expect to be able to administer if calcitriol were

administered daily may be attributable to the intermittent schedules of calcitriol employed and/or the effects of either dexamethasone or paclitaxel in modulating calcitriol-induced hypercalcemia.

We have also proposed a phase I/II clinical trial in men with hormone refractory prostate cancer involving calcitriol, dexamethasone, docetaxel and zoledronic acid. This trial will determine the maximum tolerated dose of calcitriol when given in combination with docetaxel 75 mg/m^2 + dexamethasone + zoledronic acid; examine the effect of calcitriol administration on docetaxel pharmacokinetics; conduct a phase II evaluation of this combination in patients with hormone refractory prostate cancer; and characterize the toxicity of the combination of calcitriol in combination with docetaxel and zoledronic acid in patients with AIPC. Docetaxel will be administered at a dose of 75mg/m^2, q3weeks, zolendonic acid will be adminstered at a dose of 4mg/m^2 , dexamethasone at 8 mg QDX5 and calcitriol 10μg QDX3 (starting dose). Calcitriol will be escalated to 14, 18, 26, 30 μg with further escalations at 30% increments with the following schedule:

Treatment Schedule, cycle #1 Only

	D1	D2	D3	D4	D5	D6
Docetaxel			X			
Dexamethasone	X	X	X	X	X	
Zoledronic Acid	X					
Calcitriol				X	X	X
Pk sampling			X			

Treatment Schedule, cycle #2 and thereafter

	D1	D2	D3	D4	D5	D6	
Docetaxel				X			
Dexamethasone	X	X	X	X	X		
Zoledronic Acid	X						
Calcitriol		X	X	X			
Pk sampling				X			

Patients will be treated until there is unacceptable toxicity, evidence of progressive disease, or a total of six cycles in the case of stable disease. Approximately 20-30 patients will be accrued to the phase I portion of this study and 14-32 patients on the phase II portion, depending on the response. The trial is under review and will be open late 2000.

Methods utilized:

Tumor cells and model systems. The human prostatic adenocarcinoma cell line PC-3 was obtained from the American Type Culture Collection (Manassas, VA). PC-3 cells are VDR+, androgen-independent and p53 null. For in vitro studies, cells were grown in F-medium supplemented with 10% FCS and 2 mM L-glutamine. In vivo, tumors were routinely produced by subcutaneous inoculation of 2 × 106 log-phase tissue culture cells mixed 1:1 with Matrigel in the right flank of nude mice (Taconic Farms, Germantown, NY). Studies were initiated 8 days later when tumors were palpable.

SCCVII/SF is a moderately well differentiated squamous cell carcinoma derived from a spontaneously arising tumor of the C3H mouse (15). Murine SCCVII/SF squamous cell carcinoma cells were obtained from Dr. Karen Fu (University of San Francisco, CA). They were transplanted as previously described in 6- to 10-week old female C3H/HeJ mice (obtained from The Jackson Laboratory, Bar Harbor, ME). For in vitro studies, cells were grown in RPMI-1640 medium plus 15% FCS (HyClone Laboratories, Inc., Logan, UT) and passed only twice before being returned to the animals. In vivo, tumors were routinely produced by subcutaneous

inoculation of 5 × 105 log-phase tissue culture cells in the right flank of each mouse. Studies were initiated approximately 9 days later when the tumors were palpable.

Chemicals and reagents. Calcitriol (Hoffmann-LaRoche, Nutley, NJ) was reconstituted in 100% ethyl alcohol and stored protected from light under a layer of nitrogen gas at -70 C. All handling of calcitriol was performed with indirect lighting. Paclitaxel (Bristol-Myers Squibb, Princeton, New Jersey) was purchased as a 6 mg/ml solution in Cremophor EL and was diluted in tissue culture medium or sterile saline just prior to use. The antibodies used in these studies were monoclonal mouse anti-PARP (Enzyme Systems, Livermore, CA) and monoclonal mouse anti-human Bcl-2 (DAKO Carpinteria, CA). Monoclonal anti-caspase-3 and polyclonal rabbit anti-p21 were from PharMingen (San Diego, CA). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Life Sciences (Arlington Heights, IL) and Promega (Madison, WI), respectively.

Clonogenic tumor cell survival assays. Tumor cells were incubated for 24 hours in T25 flasks (Corning Costar Corp., Cambridge, MA) with or without calcitriol. Cells were then either left untreated, or were treated with various concentrations of paclitaxel for an additional 24 hours. The cells were then harvested, counted, and re-plated at various dilutions into 6-well tissue culture plates (Corning Costar). After a 7-day incubation at 37 C in a humidified atmosphere containing 5% CO2, cell monolayers were washed with saline, fixed with 100% methanol, and stained with 10% Giemsa. Colonies were counted with the use of a light microscope. To calculate the surviving fraction, the cloning efficiency of treated cells was divided by the cloning efficiency of untreated, control cells.

In vivo clonogenic assay. The in vivo effect of paclitaxel with and without calcitriol on clonogenic tumor cells was determined by a modification of the in vivo clonogenic cell survival assay as described previously (16). Briefly, mice with 9-day squamous cell carcinomas (three to five animals per group) were treated with saline or 2.5 (g calcitriol each day for 3 days. On day 3, mice also received varying doses of paclitaxel. Twenty-four hours after the last injection, the animals were sacrificed and their tumors excised. Aliquots of minced tumor were enzymatically dissociated for 60 minutes at room temperature with a mixture of type I collagenase, deoxyribonuclease, and EDTA. Viable tumor cells were then plated at various dilutions in 6-well tissue culture plates. After incubation for 7 days, colonies were counted and the numbers of clonogenic cells per gram of tumor was calculated. The surviving fraction per gram of tumor is defined as the number of clonogenic tumor cells per gram of treated tumor divided by the number of clonogenic tumor cells per gram of control, untreated tumor.

Tumor growth inhibition. To examine the in vivo antitumor activity of calcitriol, paclitaxel, or the combination of calcitriol with paclitaxel, treatment was initiated on animals bearing palpable PC-3 or SCC tumors. Animals were treated for 3 days with single, daily injections of saline or calcitriol. On day 3, animals also received a single intraperitoneal injection of paclitaxel. Tumor measurements were obtained using calipers prior to initiating treatment (initial tumor volume) and on the days indicated. On day 3, animals also received a single intraperitoneal injection of paclitaxel. Tumor measurements were obtained using calipers prior to initiating treatment (initial tumor volume) and every day thereafter. Tumor volumes were calculated by the following formula: volume = (length × width^2)/2.

Preparation of cell lysates and Western blot analysis. Protein was extracted from in vitro treated cells using lysis buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris, 150 mM NaCl, 0.6 mM PMSF, and 5 g/ml leupeptin). Cell monolayers were washed twice with PBS, and 200 mil of lysis buffer was added per T25 flask. Flasks were rocked for 30 min at 4 C. Lysates were tranferred to 1.5 ml Eppendorf tubes and clarified by centrifugation at 13,000 rpm for 10 min at 4 C. Proteins were quantitated in duplicate using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's directions. Protein lysates were stored at -80 C before use. Proteins were resolved on SDS-polyacrylamide gels under denaturing conditions, and then electrophoretically transferred to poly(vinylidene difluoride) (PVDF) membranes (NEN Life Science Products, Boston, MA) overnight at 4°C. At room temperature, membranes were blocked for a minimum of 1h in a 5% w/v solution of non-fat milk in TBST (10 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween-20) then incubated for 1h with primary antibody. The blots were washed 3x in TBST and subsequently incubated with secondary antibody conjugated with horseradish peroxidase for 1h. The blots were again washed and the proteins detected using Renaissance Western blot chemoluminescence reagents (NEN Life Science Products).

Cell cycle analysis. The effect of various treatments on tumor cell cycle was determined using a detergent-trypsin method to stain nuclei for DNA analysis using flow cytometry as described by Vindelov et al (17). Tumor cells were seeded in T25 flasks at varying concentrations determined to produce 80-90% confluence upon harvest after the desired incubation interval. Twenty-four hours after plating, the cells were either left untreated or were

treated with calcitriol alone, paclitaxel alone, or the combination of calcitriol plus paclitaxel for varying lengths of time at 37 C. Following treatment, the media containing floating cells was harvested, and trypsin was added to the adherent cell monolayer. The resulting single cell suspension was diluted with fresh media, pooled with the floating cells, and centrifuged for 5 min at 800 rpm. The cell pellet was resuspended in citrate buffer (250 mM sucrose, 40 mM citrate, 5% DMSO) and flash frozen in dry ice/EtOH before storage at -80 Cμ. Nuclei were subsequently isolated and stained with propidium iodide as described . The stained nuclei were filtered using 30 m nylon mesh and analyzed using a FACSTAR flow cytometer.

YO-PRO-1 Staining. Cells were harvested for YO-PRO-1 staining as described above, spun down, resuspended in PBS to a concentration of 1.0 – 1.5 x 106 cells/ml, and the percent of apoptotic cells measured using the Vybrant Apoptosis Assay Kit #4 (Molecular Probes). According to the manufacturer's instructions, 1.0 □l each of YO-PRO-1 dye and propidium iodide solutions were added to 1.0 ml aliquots of suspended cells, allowed to incubate for at least 20 min, and subsequently analyzed by flow cytometry.

Clinical Trial. In conjunction with these preclinical studies we are conducting a phase one clinical trial of paclitaxel, 80mg/sqm, administered weekly for four weeks, followed by two weeks of rest. Calcitriol is administered orally daily for three days each week starting two days prior to the weekly paclitaxel dose, in escalating doses in cohorts of three patients per dose escalation level. The starting dose for this phase one trial was 4μg QD X3, weekly. Serum calcium, phosphorus, creatinine and CBC are monitored weekly, physical exam is performed q6

weeks and antitumor effect is assessed monthly. This trial is open to patients with advanced solid tumors for whom no curative or more effective therapy is available.

Key Research Accomplishments

- In prostate models, pre-treatment with calcitriol enhances paclitaxel-mediated anti-tumor activity invitro and invivo and these effects are schedule dependent

- Weekly treatment with cycles of calcitriol (dailyX3) and paclitaxel on day 3 prolonged tumor growth inhibition in PC-3 and SCC

- Calcitriol pre-treatment reduced p21 expression and accelerated paclitaxel-induced PARP cleavage in PC-3 cells

- Calcitriol accelerates paclitaxel-induced apoptosis and calcitriol–mediated reduction in p21 is not required for the increase in paclitaxel cytotoxicity

- Calcitriol cleaves MEK in a caspase-dependent manner

- Calcitriol inhibits Akt survival signaling, induces MEKK-1 expression and inhibits MEK-Erk1/2

- Calcitriol and not cisplatin induces significant loss of MEK, MEK cleavage and up-regulation of MEKK-1

- Paclitaxel and bisphospphonates (zolendonic acid) decrease calcitriol-mediated hypercalemia

- Patients with advanced cancer are treated with weekly cycles of calcitriol (dailyX3) plus paclitaxel (on day3) and have been evaluated for toxicity, determination of MTD and pharmacokinetic effects. To date, the MTD has not been reached and the trial continues to accrue.

- No dose limiting toxicity has been encountered at 38μg dose of calcitriol po QDX3 and weekly paclitaxel

- No change in peak concentration, AUC or T1/2 has been noted to date in the two schedules (DDD-paclitaxel and paclitaxel-DDD)

Reportable Outcomes

Manuscripts:

P.A. Hershberger, W.D. Yu, R.A. Modzelewski, R. M. Rueger, Z. R. Shurin, **C.S. Johnson**, and D.L. Trump. 1,25-dihydroxycholecalciferol (1,25-D_3) potentiates the antitumor activity of paclitaxel in murine squamous cell carcinoma and human prostatic adenocarcinoma. Cancer Res. (2001)

T.F. McQuire, D.L. Trump and **C.S. Johnson**. Vitamin D3 selectively promotes caspase-dependent cleavage of MEK, up-regulates MEKK-1 and induces apoptosis in murine squamous cell carcinoma cells. J. Biol. Chem. (in press)

Abstracts:

Yu W-D, Bernardi RJ, Hershberger PA, **Johnson C. S.,** and Trump DL. Effects of Calcitriol on the Glucocorticoid Receptor and the Role of Cross-talk in the Anti-Proliferative Effects of the Combination of Calcitriol and Dexamethasone. Proc. Amer. Assoc. Cancer Res. 41, 772 (2000).

McGuire TF, Brallier J, Trump DL, and **Johnson C.S**. 1,25-Dihydroxycholecalciferol (calcitriol) inhibits mitogen-activated protein kinase activity without significantly affecting MEK activity in Murine Squamous Cell Carcinoma Cells. Proc. Amer. Assoc. Cancer Res. 41, 428 (2000).

Rueger RM, Blum KE, **Johnson C.S.**, and Trump DL. The Bisphosphonate Zoledronate (CGP42446) Significantly Decreases Calcitriol Mediated Hypercalcemia. Proc. Amer. Assoc. Cancer Res. 41, 281 (2000).

Hershberger PA, Modzelewski RA, Rueger RM, Blum KE, Trump DL, and **Johnson C.S.** Enhanced Anti-Tumor Efficacy With Dexamethasone/Calcitriol/Cisplatin Therapy: Role of $P21^{WAF1}$. Proc. Amer. Assoc. Cancer Res. 41, 15 (2000).

Trump DL, Serafine S, Brufsky A, Muindi J, Bernardi R, Potter D, and **Johnson C.S.** High Dose Calcitriol ($1,25(OH)_2$ Vitamin D_3) + Dexamethasone in Androgen Independent Prostate Cancer (AIPC). Ann. Oncol. 19, 337a (2000).

Johnson, C.S., Egorin MJ, Zuhowski E, Parise R, Cappozolli M, Belani CP, Long GS, Muindi J, and Trump, DL. Effects of High Dose Calcitriol (1,25 Dihydroxyvitamin D3) on the Pharmacokinetics of Paclitaxel or Carboplatin: Results of Two Phase I Studies. Ann. Oncol. 19, 210a (2000).

Presentations:

Invited Speaker: Donald L. Trump, M.D. Seventh Annual Brown University Symposium on Vitamin D, Providence, Rhode Island, September 29, 2000 "Vitamin D in the Treatment of Cancer Clinical Studies."

Invited Speaker: Donald L. Trump, M.D. CaPCURE Seventh Annual Scientific Retreat Program, Lake Tahoe, Nevada, September 22, 2000 "Clinical and Preclinical Evaluation of 1,25 Dihydroxyvitamin D (Calcitriol) in Combination with Dexamethasone, Bisphosphonates and Cytotoxic Agents in Advanced Prostate Cancer.

Invited Speaker: Donald L. Trump, M. D. Eleventh Workshop on Vitamin D, Nashville, Tennessee, May 28, 2000 "Vitamin D and Vitamin D Analogues; Clinical development in anticancer applications."

Patents:

"Use of Pretreatment Chemicals to Enhance Efficacy of Cytotoxic Agents".

Johnson, C.S. and Trump, D.L, Inventors.

US Patent # 6,087,350.

European Patent #1 030 670.

Individuals whose salaries were partially paid by DAMD 17-98-1-8549:

Donald L. Trump	Robert Rueger
Candace S. Johnson	Terry McGuire
Doug Potter	Jill Tauch
Josh Muindi	Amy Gedeon

Conclusions

Based on epidemiologic findings, Schwartz and Hulka proposed a protective role for calcitriol in prostate cancer (25). Subsequently, the antiproliferative activity of calcitriol on prostatic adenocarcinoma cell lines in vitro (1, 2, 11) and in vivo (1, 26) was demonstrated. Antiproliferative effects of calcitriol were also observed in a pilot clinical trial in which, in a small set of patients with early, recurrent prostate cancer, calcitriol decreased the rate of PSA rise, resulting in an increase in PSA doubling times (27). PSA responses have also been observed in our ongoing phase II trial of calcitriol plus dexamethasone (dex) in hormone refractory prostate cancer (28). In this trial, 8-12 μg calcitriol is given orally Monday, Tuesday, and Wednesday each week with 4 mg dex given Sunday, Monday, Tuesday, and Wednesday. Among evaluable patients, 21% experienced a greater than 50% decrease in PSA, and 79% experienced a decrease in PSA velocity. In a further effort to develop new calcitriol based therapies for advanced malignancy, we investigated the effect of combining calcitriol with cytotoxic agents. Preclinically we demonstrate that there is an increase in anti-tumor activity in prostatic adenocarcinoma using calcitriol in combination with paclitaxel in vitro and in vivo as measured in clonogenic assays and tumor growth inhibition studies. Based upon these findings, we propose that calcitriol plus paclitaxel combination therapy may have utility in the treatment of patients with prostate cancer.

The clinical use of calcitriol may be restricted by its dose-limiting toxicity, hypercalcemia. However, a variety of calcitriol analogs, including ILX-23-7553 and EB1089, have been described which possess antiproliferative activity in vivo without inducing

hypercalcemia (7,29). It has recently been shown that EB1089, when combined with paclitaxel, inhibits the growth of MCF-7 breast cancer cells in vivo (30). Further, we observe that paclitaxel appears to attenuate calcitriol mediated hypercalcemia in pre-clinical models and our ongoing phase 1 clinical trial (31). Agents that disrupt or stabilize microtubules can inhibit calcium transport, which may account for this activity (32). Thus, paclitaxel and either calcitriol or analogs may be a safe and effective combination in the treatment of human cancer.

We have further demonstrated that calcitriol enhances paclitaxel antiproliferative activity in vitro and in vivo in the murine squamous cell model, SCCVII/SF. A previous report indicates that these cells are relatively resistant to paclitaxel in vivo at a concentration of 40 mg/kg (33). We found that although paclitaxel (20 mg/kg) has little activity when administered to tumor-bearing mice as a single agent, pre-treatment with calcitriol yields substantial antitumor activity (Fig. 3B). These data suggest that calcitriol and paclitaxel combination therapy may be useful even in the treatment of tumors that are paclitaxel insensitive.

Paclitaxel cytotoxicity is increased in MCF-7 breast cancer cells and HCT116 colon cancer cells when p21 expression is specifically perturbed (17, 18). Since calcitriol treatment reduces p21 expression in the SCC model (14) and in PC-3 cells (Fig. 5), we hypothesized that calcitriol might enhance the antitumor activity of paclitaxel via its effect on p21. To test this, we examined whether paclitaxel effects were enhanced in cells pre-treated with calcitriol (p21 low) vs cells which received concurrent calcitriol (baseline p21) and paclitaxel.

Paclitaxel-mediated apoptosis in LNCaP and PC-3 prostate cancer cells has been associated with Bcl-2 phosphorylation and inactivation (20) and/or down-modulation of the related apoptotic suppressor, Bcl-X_L (34). Consistent with these results, we found that within 24h, paclitaxel treatment resulted in phosphorylation of the apoptotic suppressor protein, Bcl-2. Loss or inactivation of Bcl-2 in prostate cancer cells following paclitaxel administration has been proposed to promote cell death by shifting the intracellular balance of death regulators in favor of pro-apoptotic molecules such as Bax (20). In our studies, paclitaxel-mediated changes in the intracellular levels of Bcl-2 temporally precede the loss of full-length PARP, suggesting that they may initiate the apoptotic program.

Although calcitriol had no effect on paclitaxel modulation of Bcl-2, calcitriol accelerated paclitaxel-induced apoptosis in cells that detached following treatment (Fig. 6B). This was most evident after 48h, where addition of calcitriol prior to or concurrent with paclitaxel resulted in a greater reduction in full-length PARP than administration of paclitaxel alone. Since the enhancement of paclitaxel effect did not depend on the schedule of calcitriol administration, it is unlikely that calcitriol increased paclitaxel activity in PC-3 via its effects on p21.

Wang et al. recently demonstrated that calcitriol pre-treatment increases paclitaxel induction of cell death and paclitaxel antitumor activity in vitro in MCF-7 breast cancer cells (35). However, in contrast to our findings, calcitriol modestly increased the effect of paclitaxel on Bcl-2 phosphorylation. Comparison of these two studies reveals that although a 24h exposure to 100nM paclitaxel results in minimal Bcl-2 phosphorylation in MCF-7, this exposure results in

strong induction of Bcl-2 phosphorylation in PC-3. This strong induction may have prohibited the detection of a subtle effect of calcitriol on paclitaxel-mediated Bcl-2 phosphorylation in PC-3.

Studies described above strongly suggest that calcitriol induces programmed cell death in SCC cells via the induction of the pro-apoptotic signaling molecule MEKK-1 while blocking pro-survival signals from the MEK-Erk and Akt pathways. Based on these findings, we propose that, prior to commitment to apoptosis, calcitriol up-regulates MEKK-1 in non-apoptotic, attached cells , but at levels which are insufficient to overcome the opposing effects of the MEK-Erk and Akt survival pathways. Unknown factors then trigger limited activation of caspases, including an unidentified caspase (or caspase-dependent protease; discussed below) that is selectively activated in calcitriol-treated cells. This caspase activity promotes MEK cleavage and removal of the MEK-Erk pro-survival signal. Furthermore, caspase-dependent proteolysis of Akt kinase occurs (24) and results in abrogation of this survival signal as well. Finally, MEKK-1 undergoes partial proteolysis at its N-terminal regulatory domain, producing species that exhibit constitutive activity, further activating caspases and significantly enhancing the pro-apoptotic signal. Activation of the MEKK-1-mediated stress pathway(s), without the presence of offsetting anti-apoptotic signals, is proposed to be sufficient for committing cells to enter apoptosis.

The results presented herein shed light on the molecular events involved in calcitriol–induced apoptosis and provide a biochemical basis for the use of calcitriol (and calcitriol analogues) in the treatment of cancer. Indeed, clinical trials are currently underway here to

assess the efficacy of calcitriol-based compounds both as a single agent as well as in combination with traditional chemotherapeutic agents. It is intriguing to speculate, based on the results described above, that calcitriol may be priming the cells to exhibit heightened sensitivity to the cytotoxic regimen by either up-regulating MEKK-1 expression/activity, inducing a calcitriol-selective caspase/protease, or both.

In summary, our data demonstrate that calcitriol enhances paclitaxel antitumor activity in PC-3 and SCC cells in vitro and in vivo and indicate that novel calcitriol/ paclitaxel based combination therapies may have significant clinical utility in the treatment of a variety of solid tumors including prostate cancer.

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Appendices: Figures for Progress Report

Manuscripts

Abstracts

Figure legends:

Fig. 1. Dose-response curves of SCC cells treated in vitro with calcitriol (1,25-D_3) alone (▲), with varying doses of paclitaxel alone (O), pre-treated with 1,25-D_3 followed by paclitaxel (●), or treated simultaneously with 1,25-D_3 plus paclitaxel (Δ), as measured by growth inhibition in the 7-day in vitro clonogenic assay. In pre-treated cells, 1,25-D_3 was added 24h prior to paclitaxel. Cells were exposed to paclitaxel for 24h. 1,25-D_3 was used at a concentration of 4 nM. Each point represents the mean surviving fraction as determined by counting triplicate wells; error bars represent the 95% confidence intervals. Note that the symbol for the 1,25-D_3 alone treatment group is arbitrarily placed with respect to the x-axis. Values for pre-treatment with 1,25-D_3 followed by paclitaxel are significantly different than those obtained for paclitaxel alone; *, $p \leq 0.01$.

Fig. 2. Dose-response curves of PC-3 cells treated in vitro with 1,25-D_3 alone (▲), with varying doses of paclitaxel alone (O), or pre-treated with 1,25-D_3 for 24h followed by paclitaxel (●) as measured by growth inhibition in the 7-day in vitro clonogenic assay. Cells were exposed to paclitaxel for 24h. 1,25-D_3 was used at a concentration of 5 μM. Each point represents the mean surviving fraction as determined by counting triplicate wells; error bars represent the 95% confidence intervals. Note that the symbol for the 1,25-D_3 alone treatment group is arbitrarily placed with respect to the x-axis. Values for pre-treatment with 1,25-D_3 followed by paclitaxel are significantly different than those obtained for paclitaxel alone; *, $p<0.0004$.

Fig. 3. 1,25-D_3 increases paclitaxel antitumor activity in SCC in vivo. (A) SCC tumor bearing mice were treated with saline (○) or 2.5 μg 1,25-D_3 each day for 3 days (●). On the third day, mice also received varying intraperitoneal doses of paclitaxel (0 to 60 mg/kg). Twenty-four hours later, tumors were harvested, dissociated, and plated in the excision clonogenic assay. Colonies were enumerated after 7 days. Each point represents the mean surviving fraction for total clonogenic cells per gram of tumor (3 to 5 mice per treatment group). (*)Values for treatment with 1,25-D_3 followed by paclitaxel are significantly different than those obtained for paclitaxel alone; $p<0.01$. (B) C3H mice bearing palpable, subcutaneous SCC tumors were treated with either saline (□), 1.25 μg 1,25-D_3 daily for 3 days (▲), 20 mg/kg paclitaxel on day 3 (○), or the combination of 1.25 μg 1,25-D_3 daily for 3 days plus 20 mg/kg paclitaxel on day 3 (●). Both agents were administered i.p. Tumor measurements were obtained on the days indicated, and fractional tumor volumes calculated as described in Materials. Data points represent the mean fractional tumor volume ± SD for 5 animals per group. Values significantly different from no treatment are shown: *, $p < .01$.

Fig. 4. 1,25-D_3 increases paclitaxel antitumor activity in PC-3 in vivo. (A) Nude mice bearing palpable, subcutaneous PC-3 tumors were treated with either saline (□), 0.75 μg 1,25-D_3 daily for 3 days (▲), 10 mg/kg paclitaxel on day 3 (○), or the combination of 0.75 μg 1,25-D_3 daily for 3 days plus 10 mg/kg paclitaxel on day 3 (●). Both agents were administered i.p. Fractional tumor volumes were calculated as described in the legend for Fig. 3B. Data points represent the mean fractional tumor volume ± SD for 5 animals per group. Arrows indicate treatment days.

(B) Animals previously treated with 1,25-D_3 plus paclitaxel on days 8-10 were treated with a second cycle of therapy on days 29-31.

Fig 5. Antitumor activity of calcitriol and docetaxel. Fractional tumor volume (mean ± SD) of PC-3 tumor bearing mice (8-10 per group) either untreated (○), treated with a single dose of docetaxel (1mg/kg) with dex (9 μg/mouse, daily x 4; -1, 1, 2, 3) (●), or pre-treated with calcitriol (0.75 μg/day x 3 days)/dex (□) and in combination (■).

Fig. 6. 1,25-D3 modulates expression of the cdk inhibitor p21 in PC-3 cells. Whole cell lysates were prepared from subconfluent PC-3 cells treated in vitro with EtOH solvent control or 5 μM 1,25-D_3 for 24-96h. Proteins were resolved on SDS-polyacrylamide gels under denaturing conditions, transferred to PVDF membrane, and analyzed by Western blot using polyclonal anti-p21 antibodies. Protein expression levels were quantitated by densitometry and are expressed as a percentage of EtOH control at each time point.

Fig. 7. Paclitaxel modulates expression of apoptosis associated proteins Bcl-2 and PARP in PC-3 cells. Whole cell lysates were prepared from subconfluent PC-3 cells treated in vitro with EtOH, 5 μM 1,25-D_3, 100 nM paclitaxel (ptx), or 5 μM 1,25-D_3 concurrent with (1,25D3+ptx) or prior to (1,25-D3/ptx) 100 nM paclitaxel. 1,25-D3 pre-treatment was for 72h. Time points are hours post paclitaxel addition. Proteins were resolved on SDS-polyacrylamide gels under denaturing conditions, transferred to PVDF membrane, and analyzed by Western blot using antibodies raised against (A) Bcl-2 or (B) PARP. (*) No floating cells were obtained.

Fig 8. Calcitriol induces apoptosis in tumor cells in vitro. Cells were treated with either vehicle (< 0.001 % ethanol, EtOH) or 10 nM calcitriol in RPMI medium containing 12 % fetal bovine serum (FBS). After 2 days, cells were processed for Western blotting. Immunoblot analysis with anti-PARP indicates that the floating population of cells (but not the attached cells) are apoptotic.

Fig 9. Calcitriol induces MEK cleavage and blocks MEK-Erk1/2 signaling. Cells were treated and processed for immunoblotting to assess the phosphorylation/activation and expression of MEK and Erk1/2 . Cleavage fragments appeared at either 33 kDa or 28 kDa in apoptotic cell populations.

Fig 10. Calcitriol-induced MEK cleavage and inhibition of MEK-Erk signaling is inhibited by zVAD. Cells were treated and lysates prepared and subjected to immunoblotting with appropriate antibodies.

Fig 11. Cells were treated 2 days and then processed for immunoblotting with anti-phospho-Akt and anti-Akt antibodies.

Fig 12. Calcitriol induces MEKK-1 expression and proteolysis in apoptotic cells. (A) cells were treated 2 d and then processed for anti-MEKK-1 immunoblotting to assess MEKK-1 expression. (B) cells were treated for either 1 or 2 d and then processed as described above.

Fig 13. Calcitriol and cisplatin both induce PARP cleavage but only calcitriol induces significant loss of MEK expression, MEK cleavage, and up-regulation/proteolysis of MEKK-1.

Cells were treated 2 d with either vehicle, 10 nM calcitriol or 1 □g/ml cisplatin (cDDP) and processed for immunoblotting to assess PARP cleavage, MEK expression/cleavage, and MEKK-1 expression/proteolysis. These results suggest that MEK cleavage is not a general phenomenon of apoptosis (as is PARP cleavage), but is selectively induced by calcitriol.

Fig 14. Absolute neutrophil counts from patients(#1-4) treated with paclitaxel and calcitriol (4μg) at various times post-treatment with calcitriol (at day 0).

Fig 15. Mean plasma paclitaxel concentrations from a representative patient on day 0 (•) and day 10 (o).

Fig16. Comparison of the AUC of paclitaxel from the two courses (DDD-paclitaxel and paclitaxel-DDD) Each dot represents results from a single patient.

Fig 17. Serum calcitriol AUC from patients treated with paclitaxel and calcitriol. Each dot represents data from an individual patient.

Figure 1

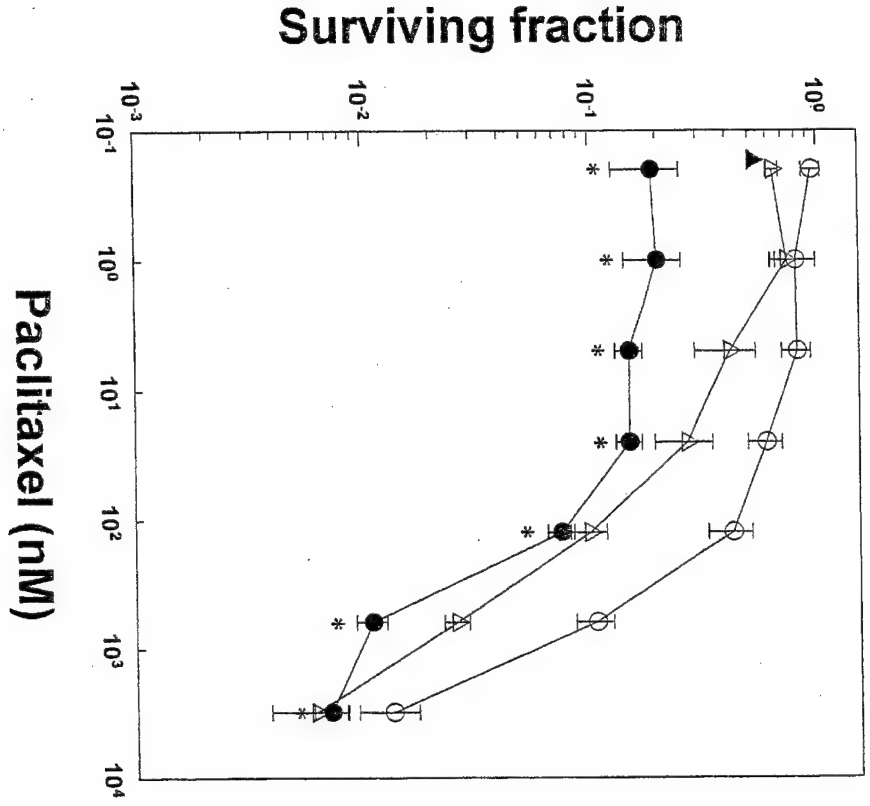

Surviving fraction
10⁻³
10⁻²
10⁻¹
10⁰
Paclitaxel (nM)
10⁻¹
10⁰
10¹
10²
10³
10⁴

Figure 2

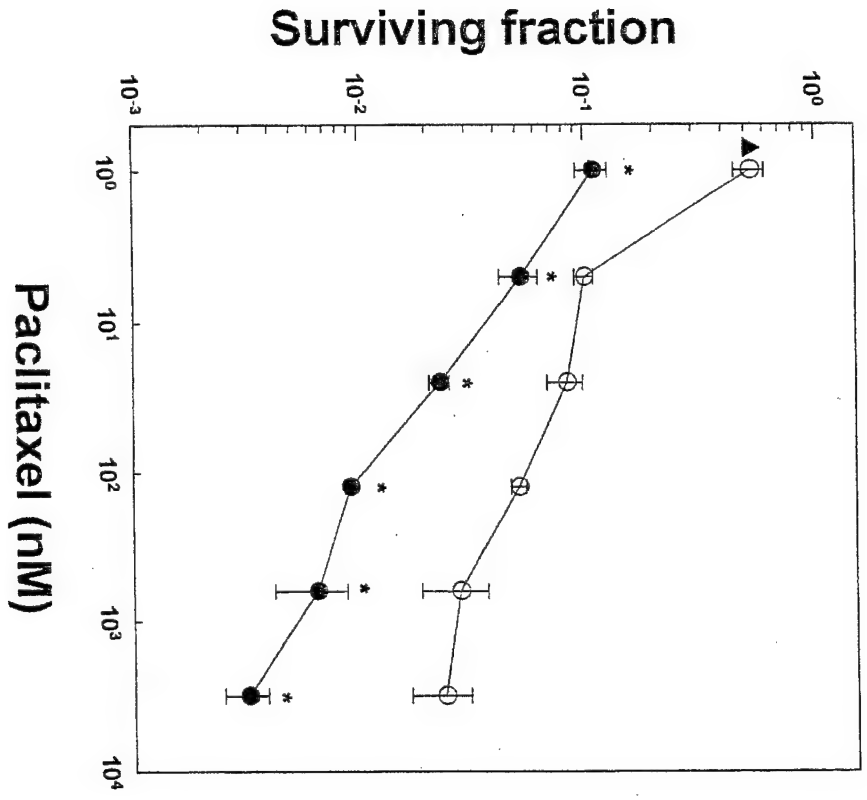

Surviving fraction
10-3
10-2
10-1
100
Paclitaxel (nM)
100
101
102
103
104
*

Figure 3 A & B

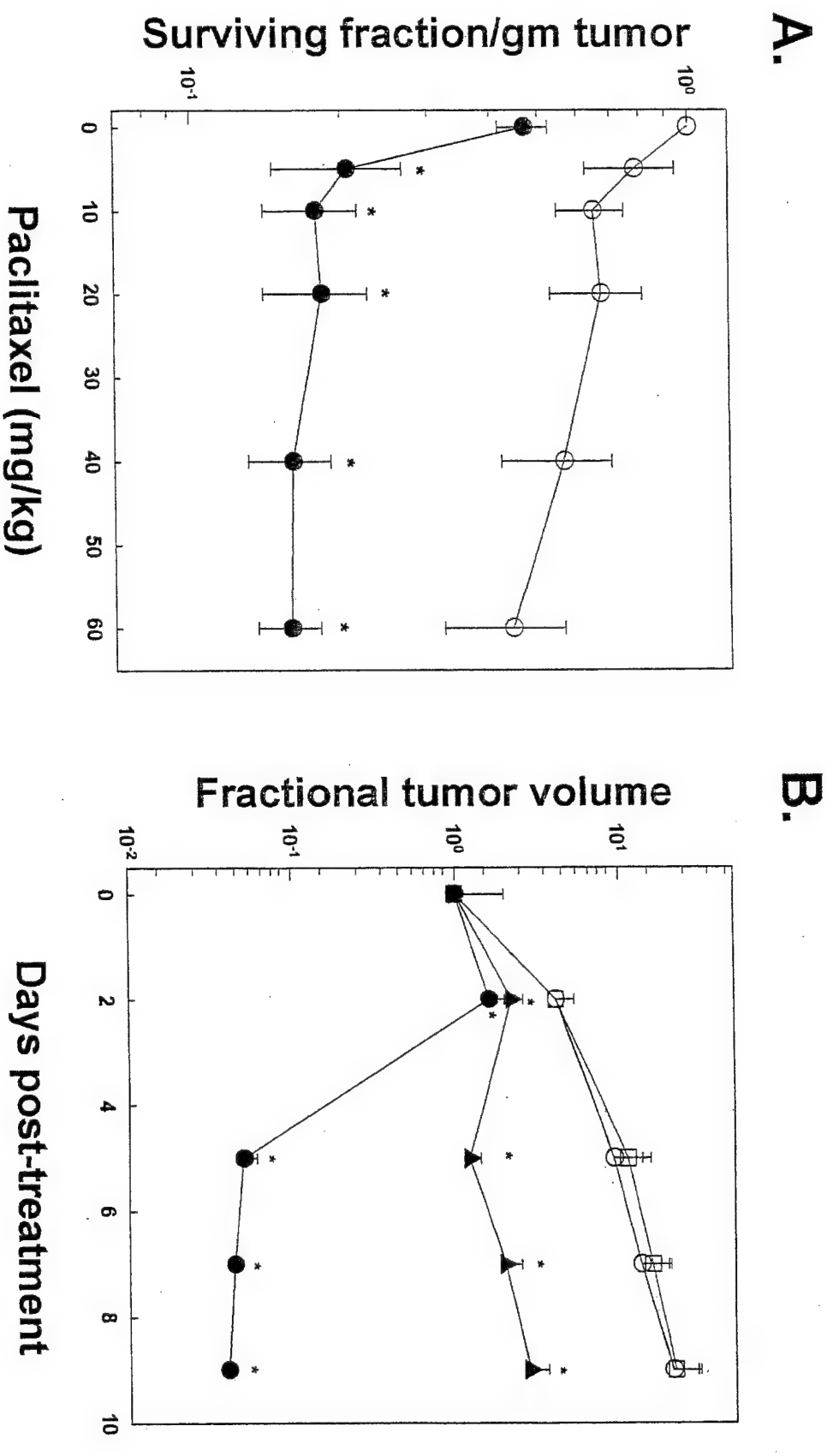

A.
Surviving fraction/gm tumor
10^{-1}
10^{0}
0
10
20
30
40
50
60
Paclitaxel (mg/kg)
B.
Fractional tumor volume
10^{-2}
10^{-1}
10^{0}
10^{1}
0
2
4
6
8
10
Days post-treatment

Figure 4 A & B

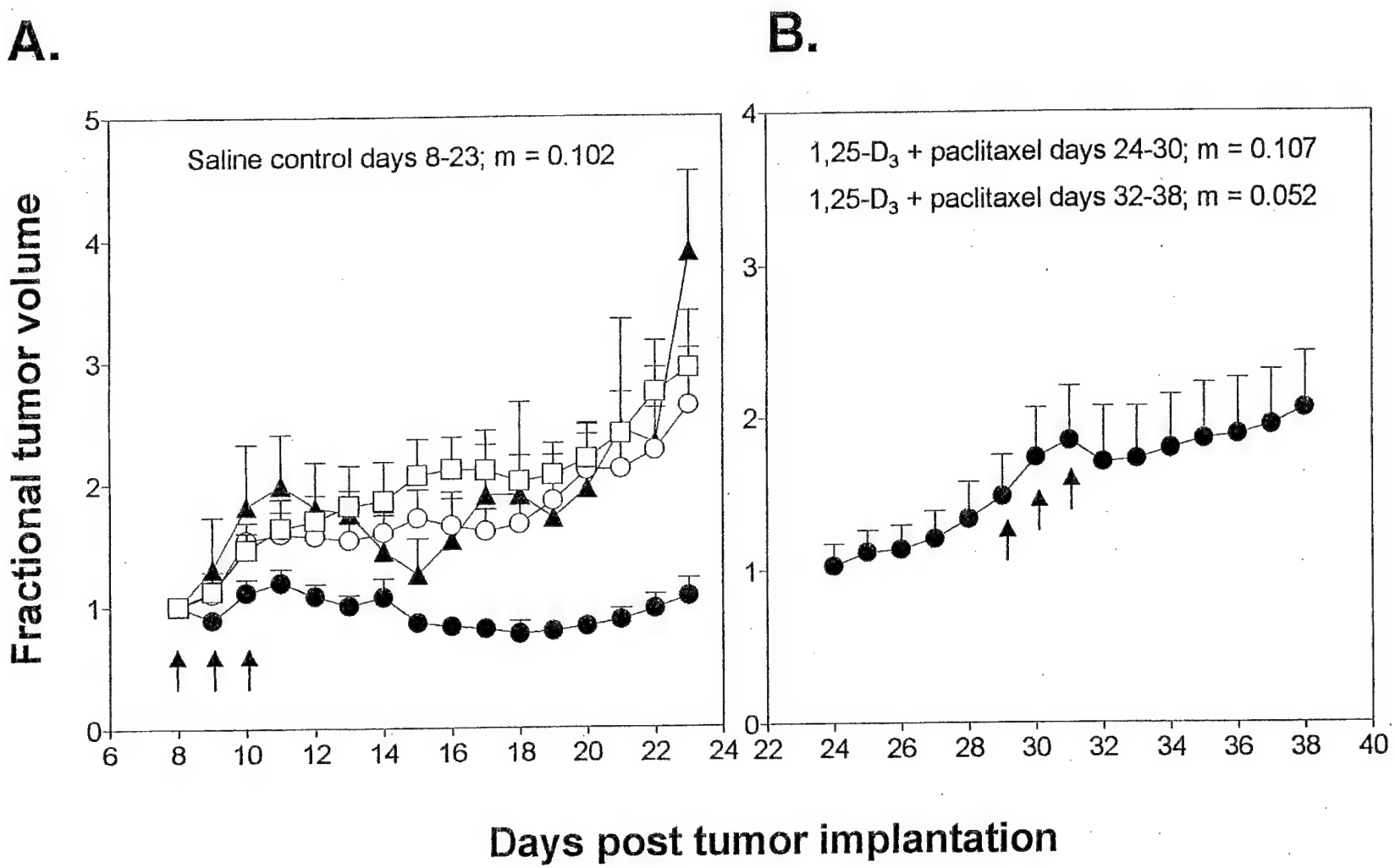

A.
B.
Saline control days 8-23; m = 0.102
1,25-D3 + paclitaxel days 24-30; m = 0.107
1,25-D3 + paclitaxel days 32-38; m = 0.052
Fractional tumor volume
Days post tumor implantation

Figure 5

Effect of 1,25D_3 and docetaxel on PC-3 tumor bearing mice.

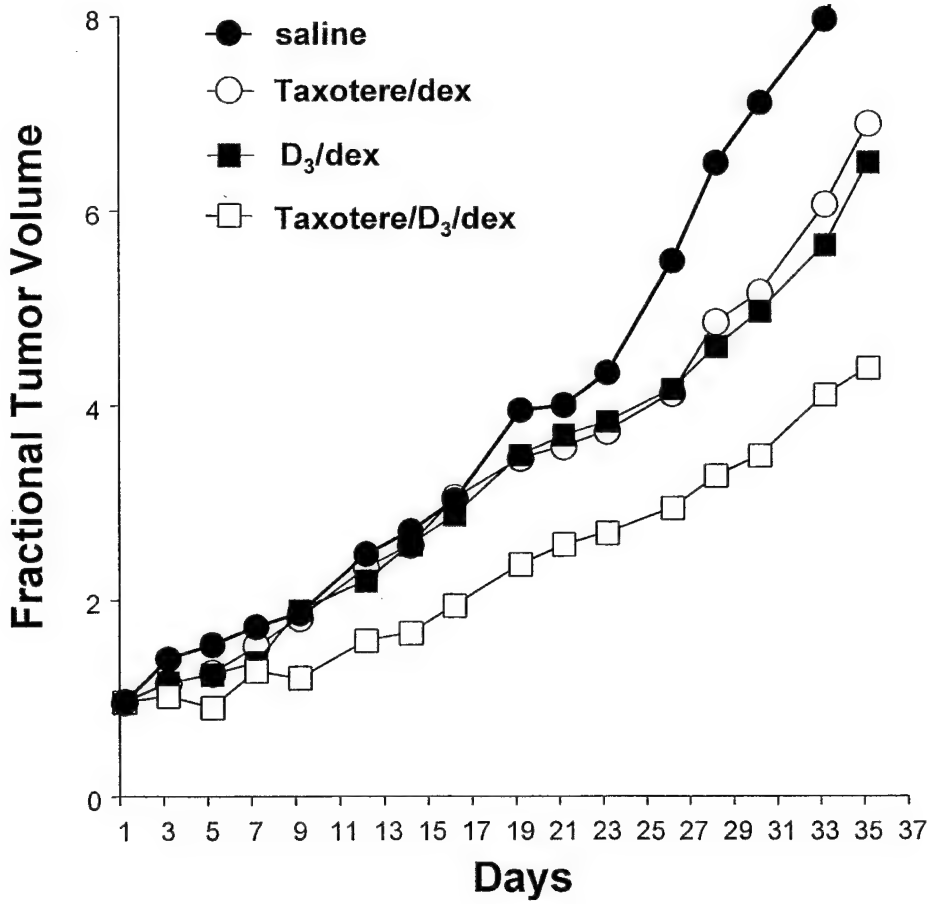


Figure 6

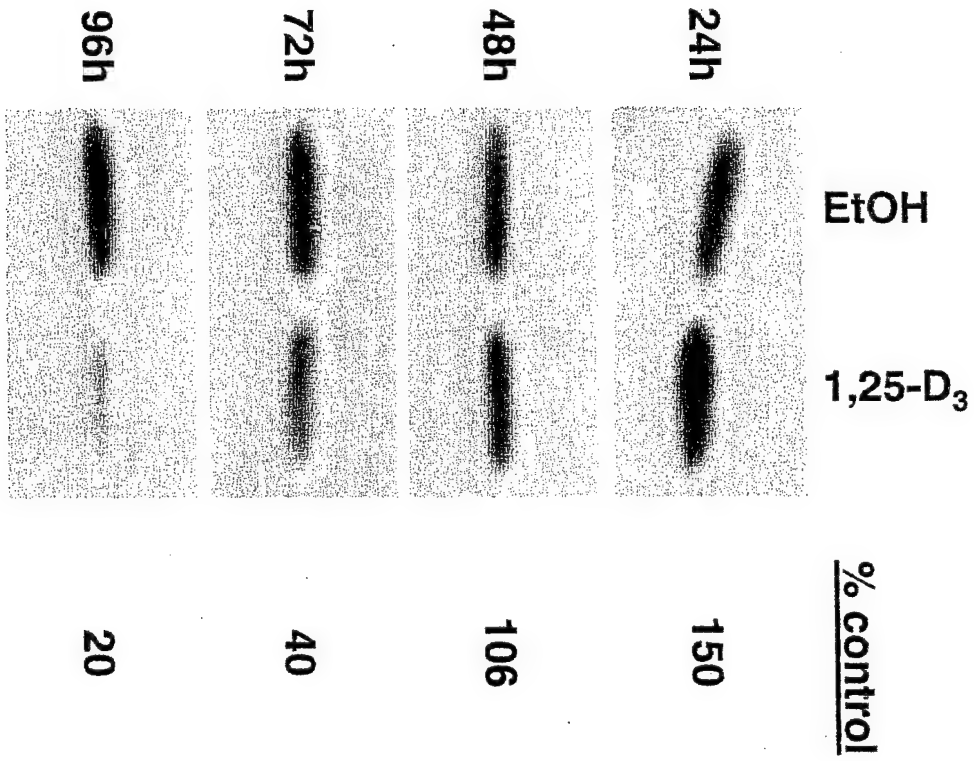


Figure 7A

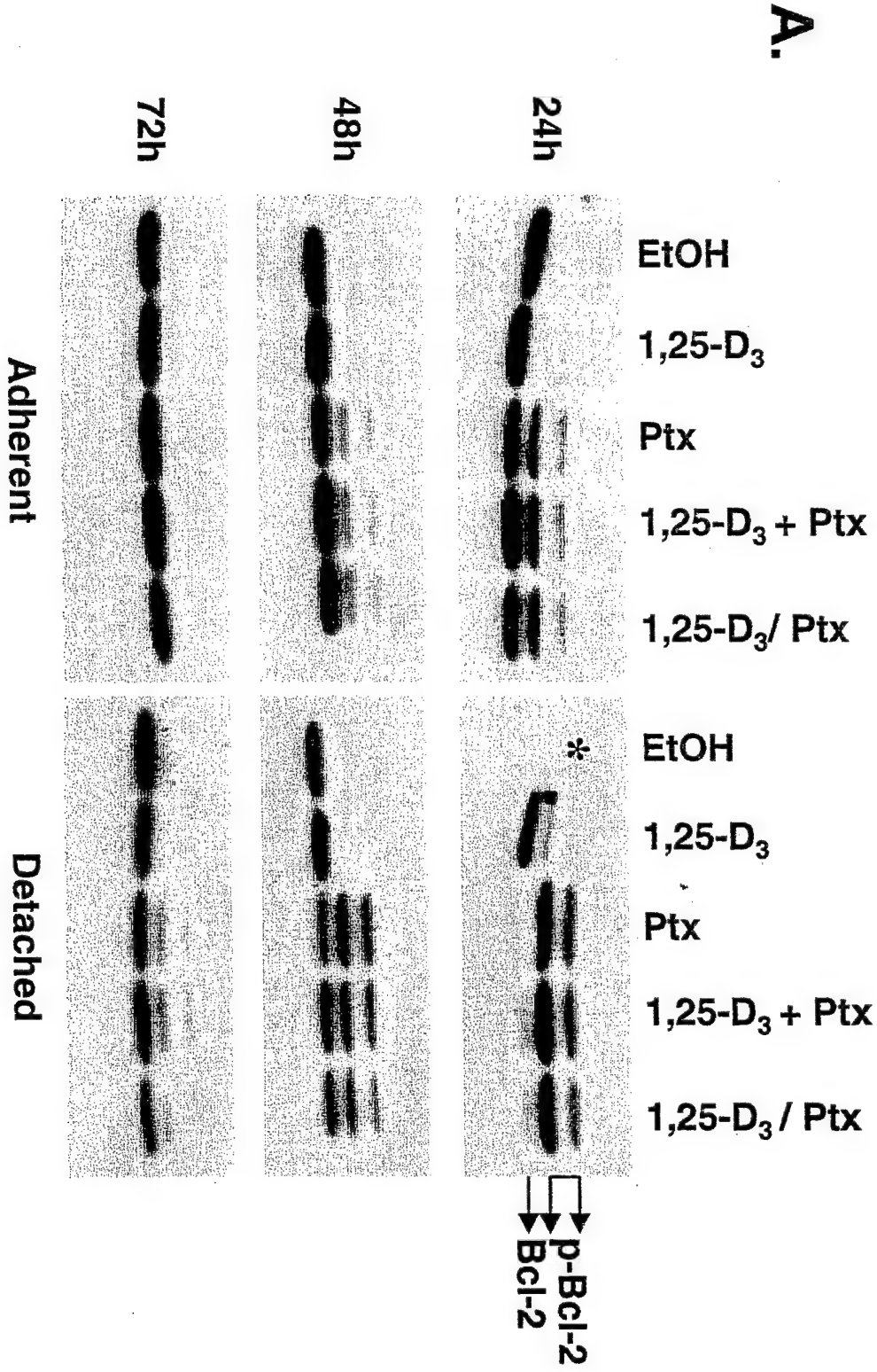
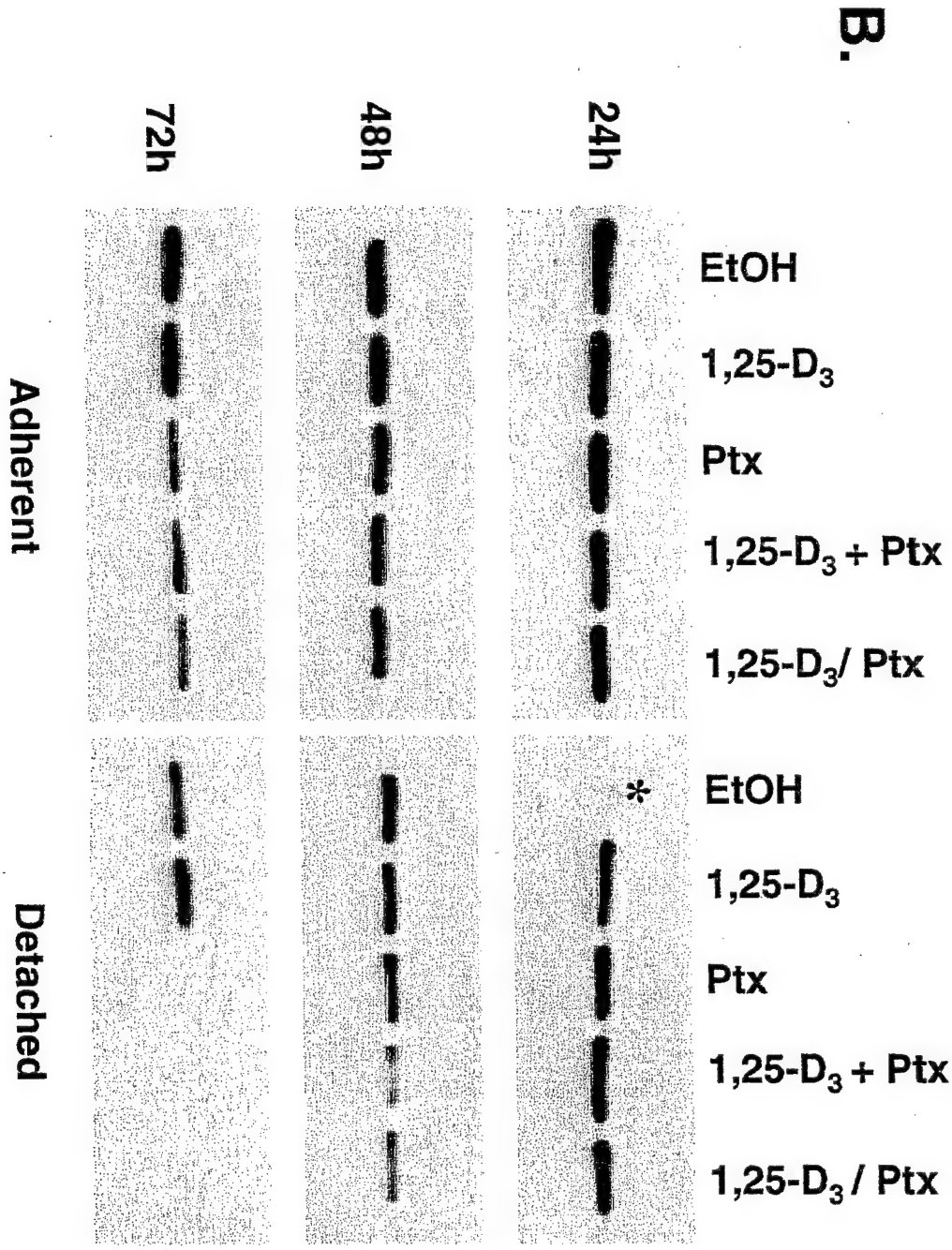
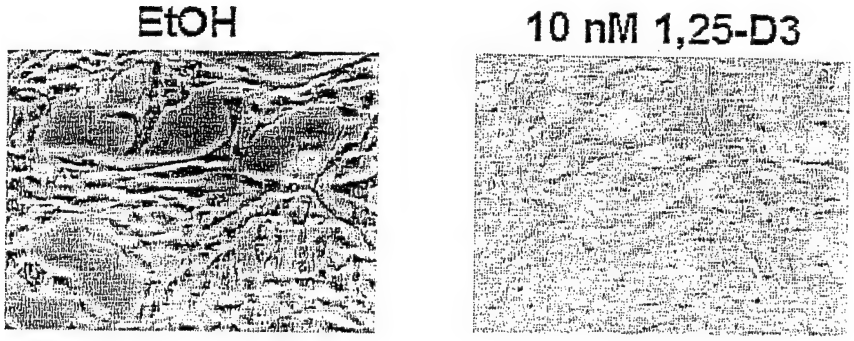

A.
72h
48h
24h
Adherent
Detached
EtOH
1,25-D3
Ptx
1,25-D3 + Ptx
1,25-D3/ Ptx
EtOH
1,25-D3
Ptx
1,25-D3 + Ptx
1,25-D3 / Ptx
*
p-Bcl-2
Bcl-2

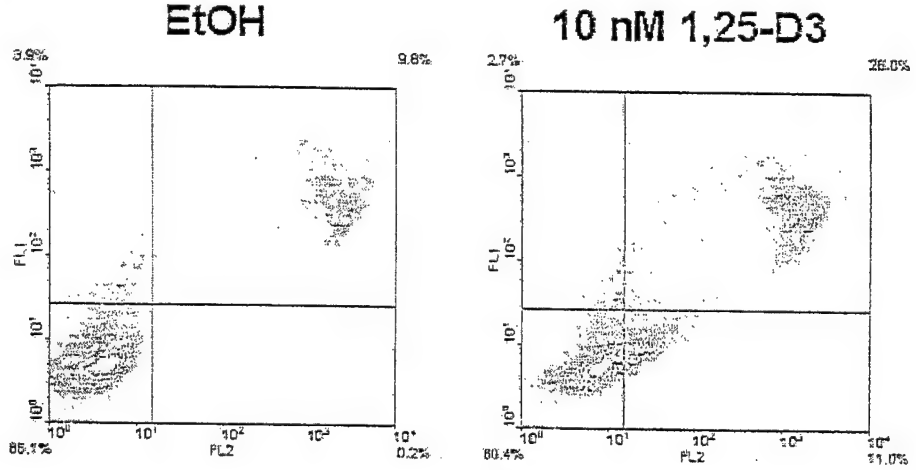
Figure 7B


B.
72h
48h
24h
Adherent
Detached
EtOH
1,25-D3
Ptx
1,25-D3 + Ptx
1,25-D3/ Ptx
EtOH
1,25-D3
Ptx
1,25-D3 + Ptx
1,25-D3 / Ptx
*

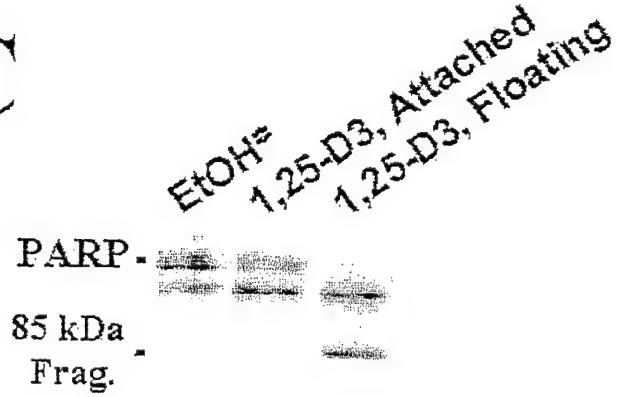
A


EtOH
10 nM 1,25-D3

B


EtOH
10 nM 1,25-D3
FL1
PL2

C


EtOH
1,25-D3, Attached
1,25-D3, Floating
PARP
85 kDa Frag.

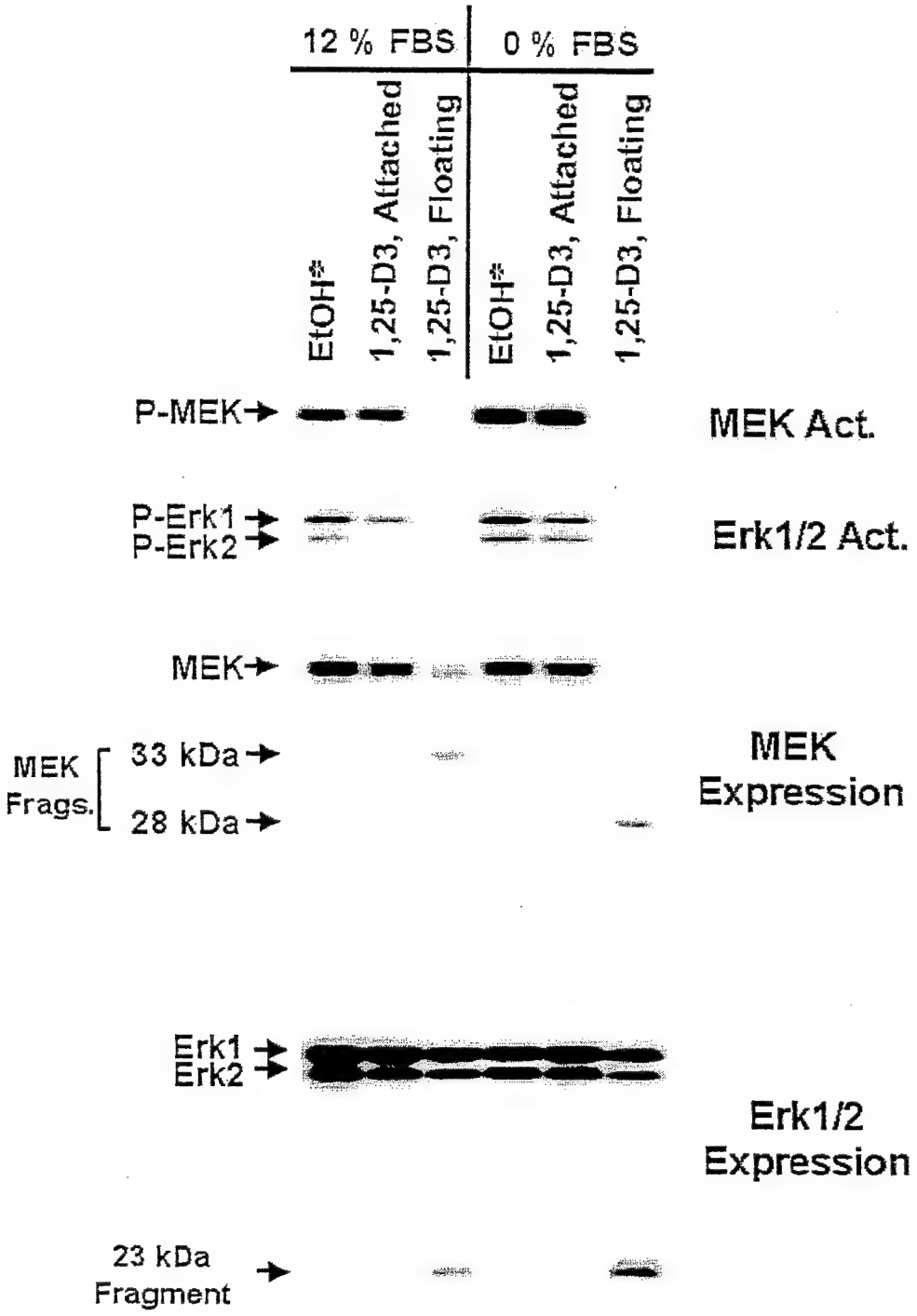
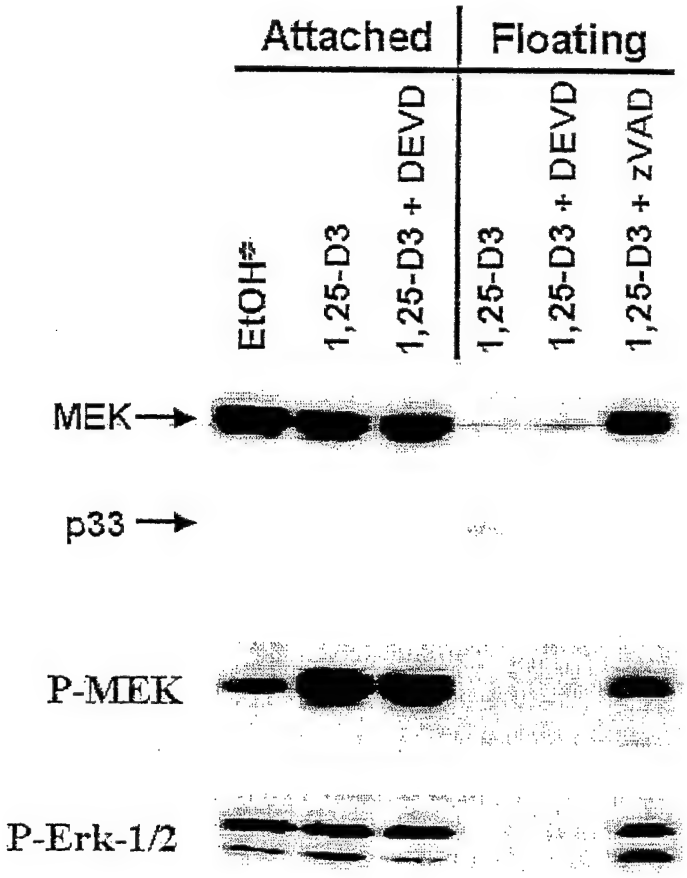
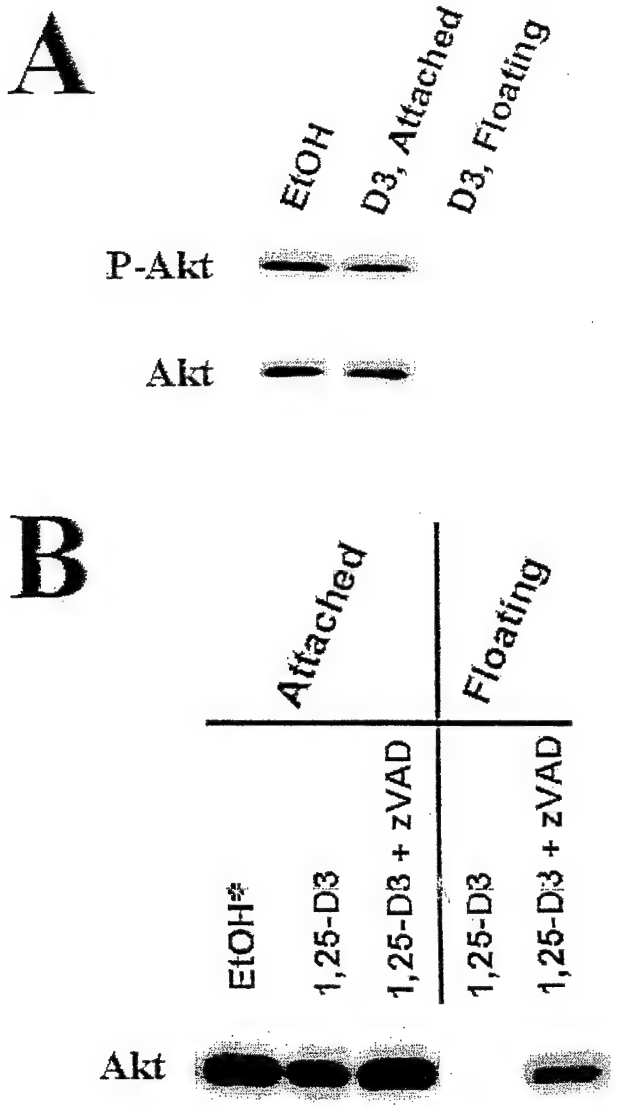
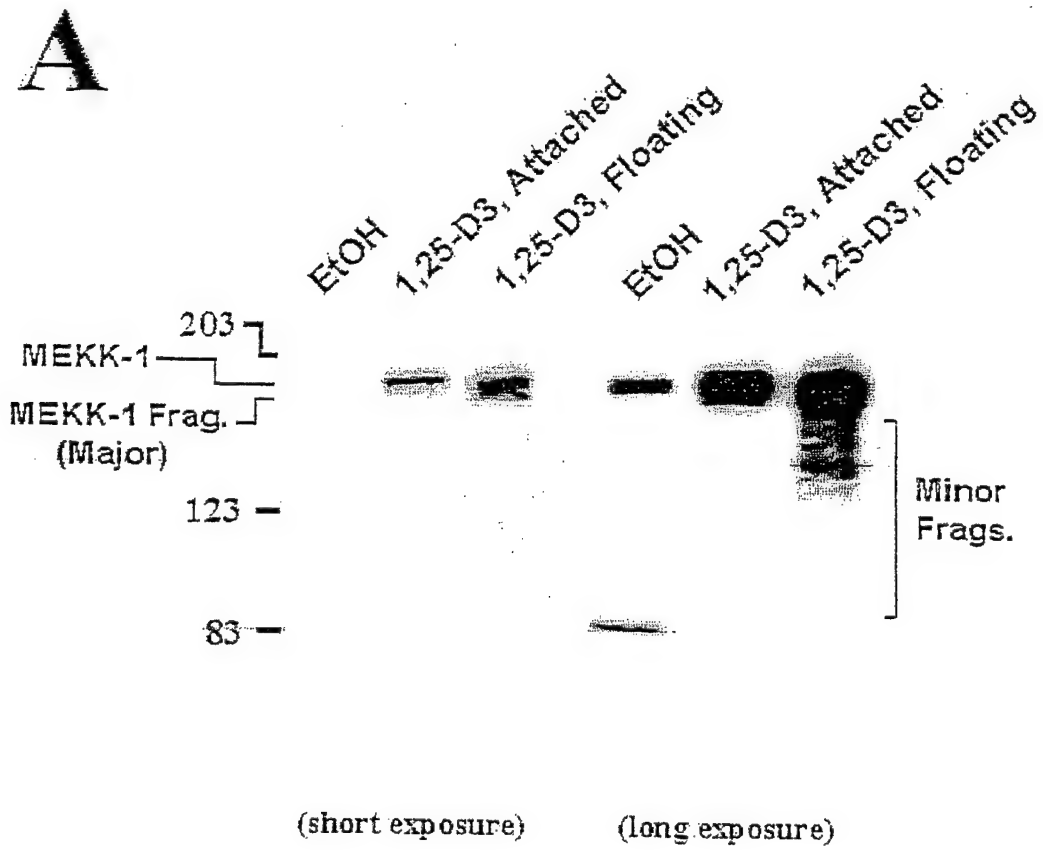

12 % FBS
0 % FBS
EtOH*
1,25-D3, Attached
1,25-D3, Floating
EtOH*
1,25-D3, Attached
1,25-D3, Floating
P-MEK
MEK Act.
P-Erk1
P-Erk2
Erk1/2 Act.
MEK
MEK Frags.
33 kDa
28 kDa
MEK Expression
Erk1
Erk2
Erk1/2 Expression
23 kDa Fragment

Figure 10


Attached
Floating
EtOH
1,25-D3
1,25-D3 + DEVD
1,25-D3
1,25-D3 + DEVD
1,25-D3 + zVAD
MEK
p33
P-MEK
P-Erk-1/2


A
EtOH
D3, Attached
D3, Floating
P-Akt
Akt
B
Attached
Floating
EtOH*
1,25-D3
1,25-D3 + zVAD
1,25-D3
1,25-D3 + zVAD
Akt


A
EtOH
1,25-D3, Attached
1,25-D3, Floating
EtOH
1,25-D3, Attached
1,25-D3, Floating
203
MEKK-1
MEKK-1 Frag.
(Major)
123
83
Minor
Frags.
(short exposure)
(long exposure)

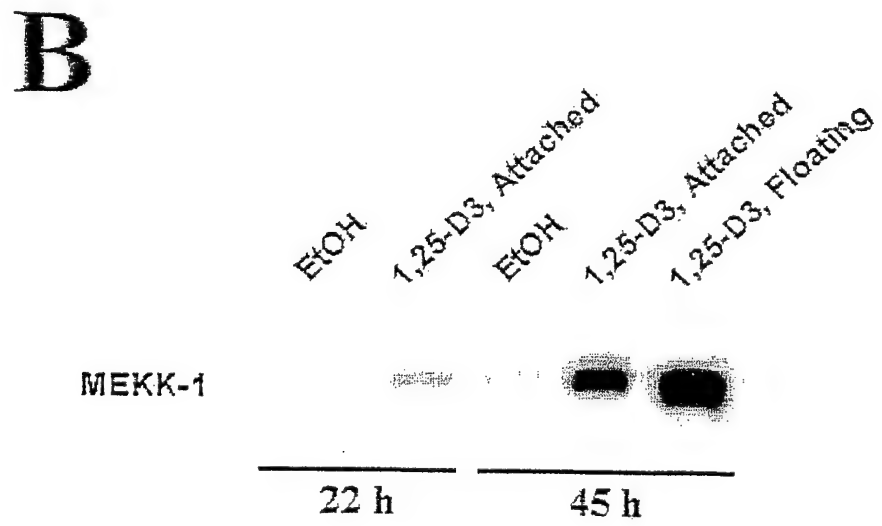

B
EtOH
1,25-D3, Attached
EtOH
1,25-D3, Attached
1,25-D3, Floating
MEKK-1
22 h
45 h

Figure 13

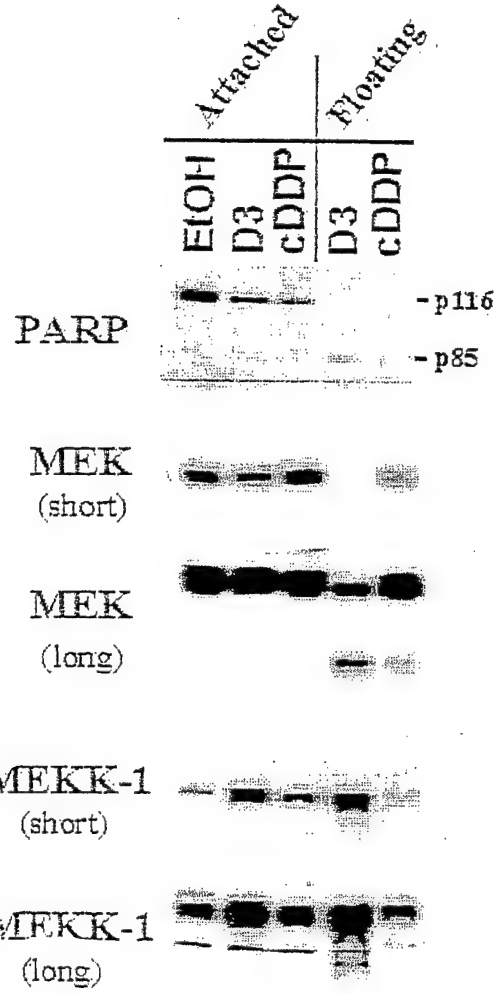

Attached
Floating
EtOH
D3
cDDP
D3
cDDP
PARP
- p116
- p85
MEK
(short)
MEK
(long)
MEKK-1
(short)
MEKK-1
(long)

Figure 14

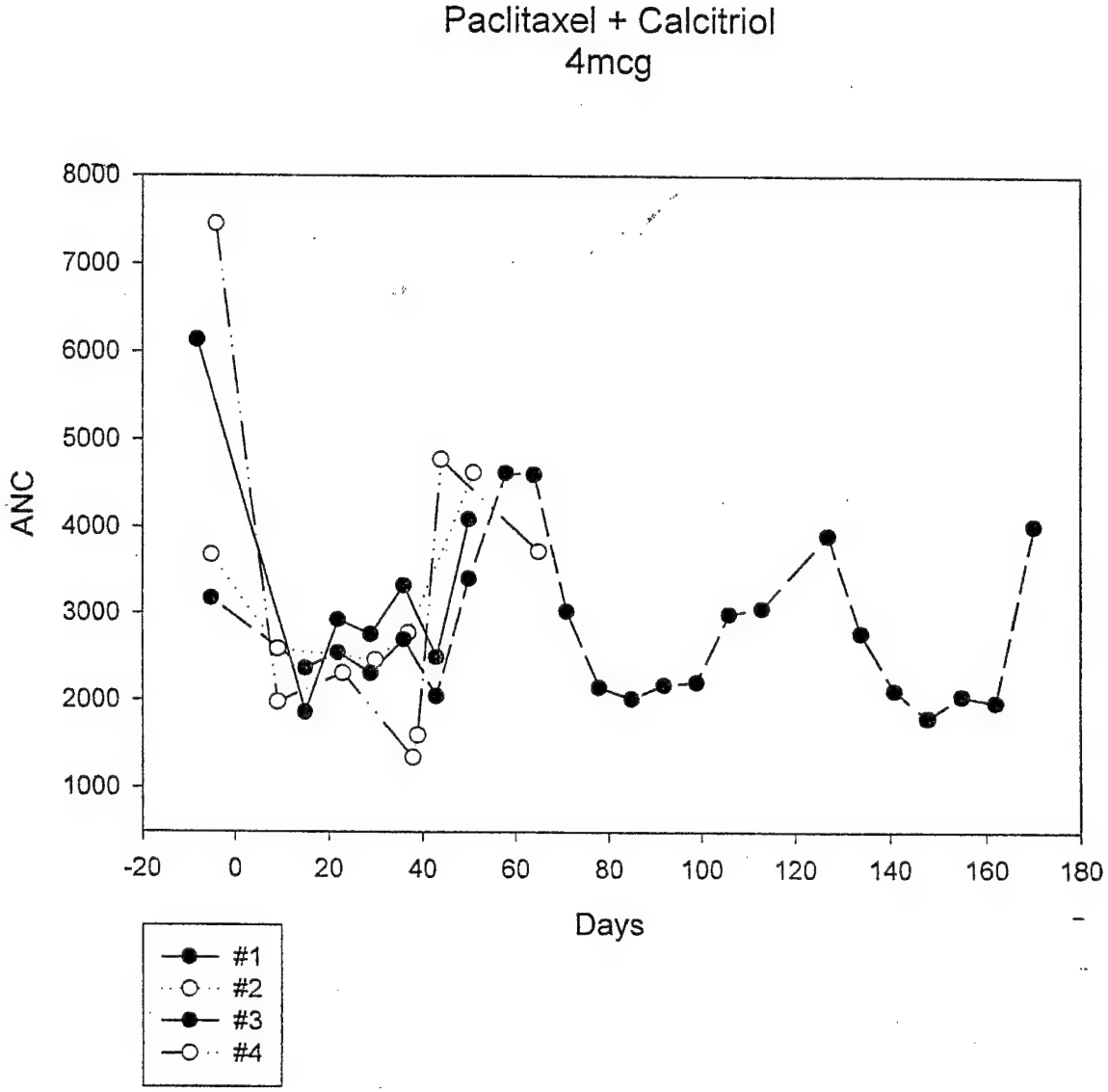

Paclitaxel + Calcitriol
4mcg
ANC
8000
7000
6000
5000
4000
3000
2000
1000
-20
0
20
40
60
80
100
120
140
160
180
Days
#1
#2
#3
#4

Figure 15

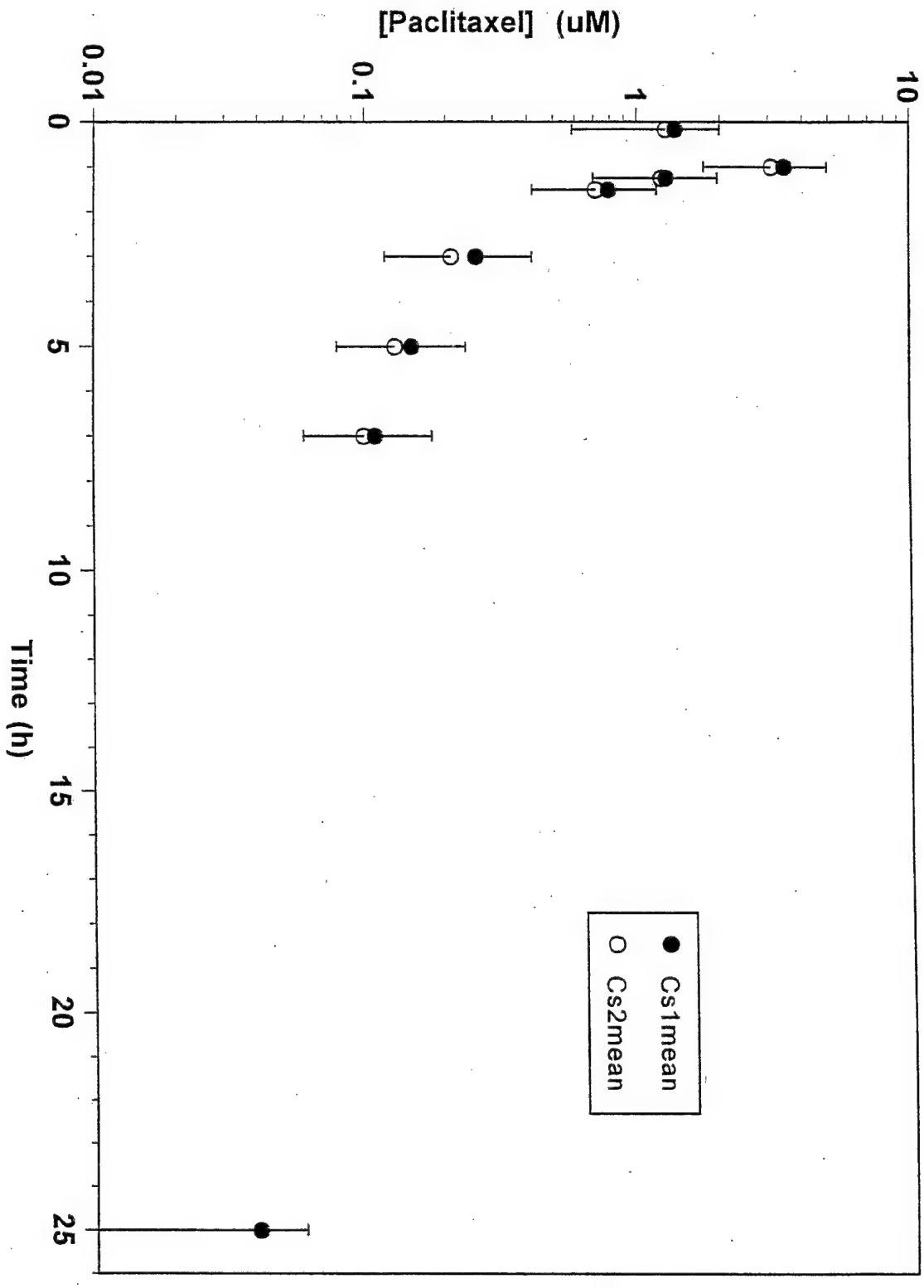

UPCI 98019 Mean Plasma Paclitaxel Concentrations
[Paclitaxel] (uM)
0.01
0.1
1
10
Time (h)
0
5
10
15
20
25
Cs1mean
Cs2mean

Figure 16

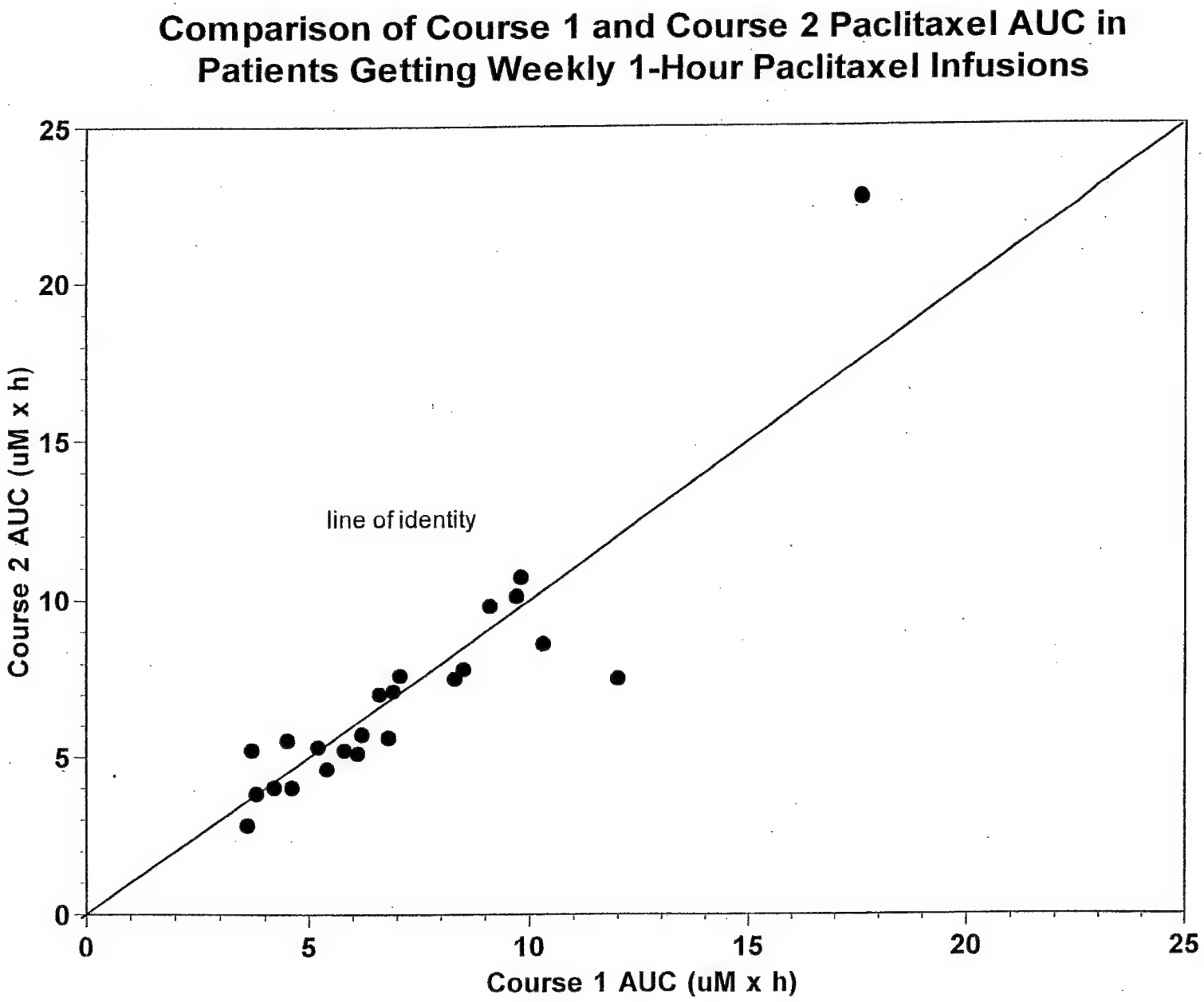
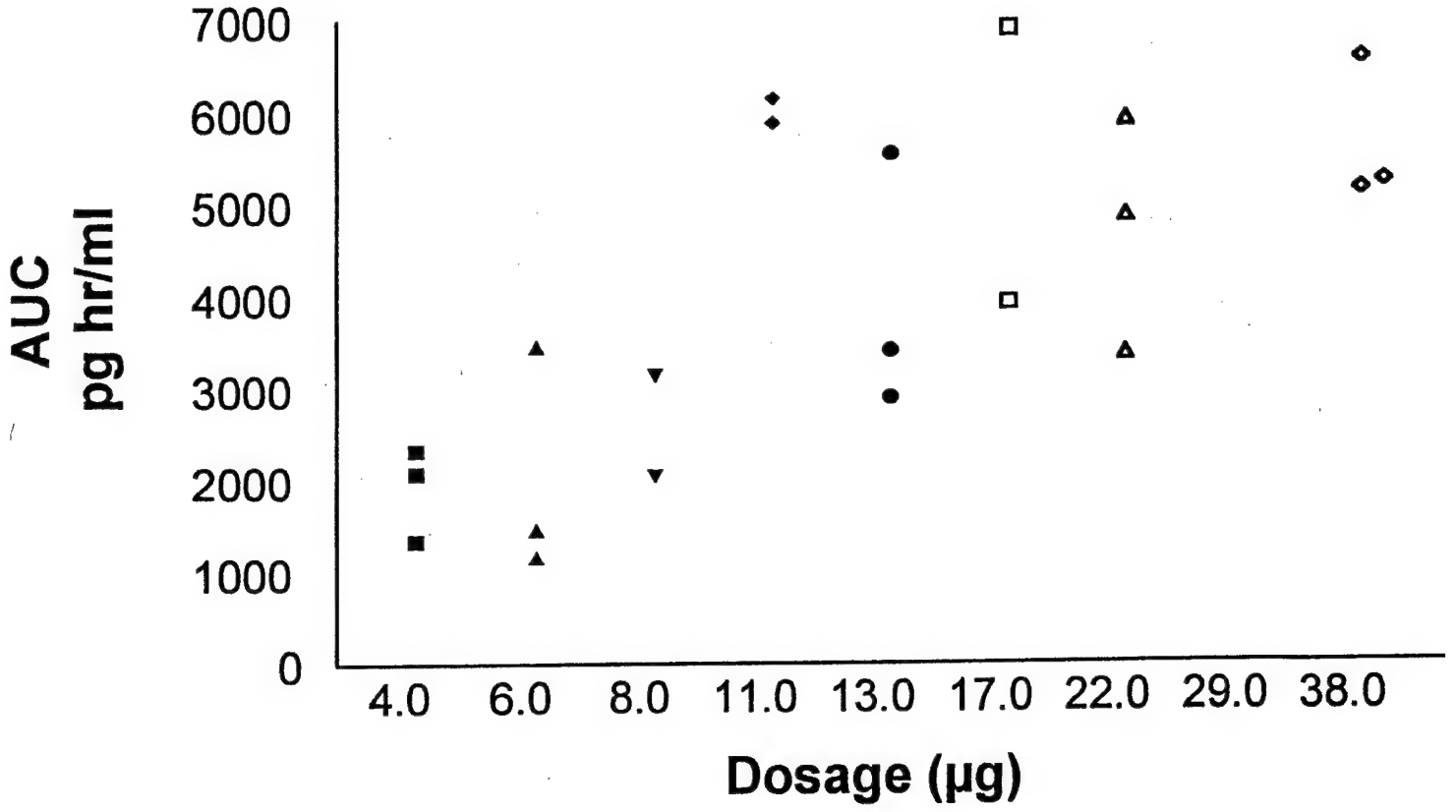

Comparison of Course 1 and Course 2 Paclitaxel AUC in Patients Getting Weekly 1-Hour Paclitaxel Infusions
Course 2 AUC (uM x h)
Course 1 AUC (uM x h)
line of identity
0
5
10
15
20
25

Figure 17

Calcitriol and Paclitaxel: Serum Calcitriol AUC



Calcitriol (1,25-Dihydroxycholecalciferol) Enhances Paclitaxel Antitumor Activity *in Vitro* and *in Vivo* and Accelerates Paclitaxel-induced Apoptosis[1]

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Departments of Pharmacology [P. A. H., W-D. Y., R. A. M., R. M. R., C. S. J.], Medicine [C. S. J., D. L. T.], and Surgery [D. L. T.] and the University of Pittsburgh Cancer Institute [C. S. J., D. L. T.], University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15213

ABSTRACT

We demonstrated that calcitriol has antiproliferative activity in squamous cell carcinoma and prostatic adenocarcinoma and enhances the antitumor activity of platinum-based agents. In this study, we examined whether calcitriol also increases paclitaxel cytotoxicity. The effect of treatment on growth of the murine squamous cell carcinoma (SCCVII/SF) and human prostatic adenocarcinoma (PC-3) was determined by clonogenic assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and monitoring tumor growth. Treatment of SCC or PC-3 cells *in vitro* with calcitriol prior to paclitaxel significantly reduced clonogenic survival compared with either agent alone. Median-dose effect analysis revealed that calcitriol and paclitaxel interact synergistically. Treatment of SCC or PC-3 tumor-bearing mice with calcitriol prior to paclitaxel resulted in substantially greater growth inhibition than was achieved with either agent alone, supporting the combined use of calcitriol and paclitaxel in the treatment of solid tumors. To explore the molecular basis for the enhanced antitumor activity of this combination, the effect of treatment on $p21^{Waf-1}$ (p21), Bcl-2, and poly(ADP-ribose) polymerase expression was evaluated in PC-3. A 72-h pretreatment with calcitriol reduced p21 expression and increased paclitaxel cytotoxicity (measured after 24 h) without evidence of apoptosis [poly(ADP-ribose) polymerase cleavage]. After 48 h, paclitaxel induced apoptosis, the extent of which was increased similarly by pretreatment or concurrent treatment with calcitriol. We therefore propose a model for calcitriol enhancement of paclitaxel cytotoxicity in which the "early" (24 h) effects are schedule dependent and not attributed to enhancement of paclitaxel-induced apoptosis. In contrast, the "delayed" (48-h) enhancement of paclitaxel activity by calcitriol is schedule independent and associated with acceleration of apoptosis.

INTRODUCTION

In addition to its classical role in bone and mineral metabolism, the seco-steroid hormone, vitamin D_3 (calcitriol, 1,25-dihydroxycholecalciferol) has antiproliferative activity in solid tumor models both *in vitro* (1–7) and *in vivo* (1, 4, 7, 8). Calcitriol binding activates the vitamin D receptor, a member of the steroid nuclear receptor superfamily, resulting in modulation of the transcription of target genes (9). Calcitriol treatment induces expression of the cdk[3] inhibitors, $p21^{Waf1/Cip1}$ (p21) and/or $p27^{kip1}$ (p27), in breast (10), prostate (11), and pancreatic cancer cell lines (6) *in vitro*. These proteins block progression into S-phase by binding to and inhibiting cyclin:cdk complexes (12).

We determined that calcitriol inhibits growth of the murine SCC SCCVII/SF (2, 7) and the growth and metastatic potential of the Dunning rat prostatic adenocarcinoma, Mat-lylu (1). We subsequently evaluated the use of calcitriol in combination with cytotoxic agents and found that calcitriol synergistically enhanced the antitumor activity of cisplatin and carboplatin *in vitro* and *in vivo*, and that these effects were schedule dependent (13).

In SCC, mechanistic studies revealed that calcitriol induces G_0-G_1 arrest (13), a decrease in Rb phosphorylation, an increase in expression of p27, and a decrease in expression of p21 (14). Expression of p21 is also reduced in SCC tumors harvested from animals treated with therapeutic doses of calcitriol (14). These findings suggest that p21 down-modulation may be a component of the mechanism by which calcitriol exerts antiproliferative activity.

Recent studies indicate that a reduction in p21 expression sensitizes tumor cells to both DNA-damaging agents (15, 16) and microtubule-damaging agents such as paclitaxel (17–19). In MCF-7 breast carcinoma cells, paclitaxel induces p21 expression; treatment of these cells with antisense p21 oligonucleo-

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[2] To whom requests for reprints should be addressed, at University of Pittsburgh, Department of Pharmacology, W1002 Biomedical Science Tower, Pittsburgh, PA 15213. Phone: (412) 648-2344; Fax: (412) 624-7737; E-mail: pah13+@pitt.edu.

[3] The abbreviations used are: cdk, cyclin-dependent kinase; SCC, squamous cell carcinoma; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly (ADP-ribose) polymerase; CI, combination index; PSA, prostate-specific antigen; PVDF, polyvinylidene difluoride.

tides increases paclitaxel cytotoxicity (17). Similarly, paclitaxel antitumor activity is increased *in vitro* and *in vivo* in HCT116 colon carcinoma cells made deficient in p21 expression (18). Compared with p21+/+ controls, these cells displayed an increase in paclitaxel-induced apoptosis.

Given the ability of calcitriol to decrease p21 expression in SCC *in vitro* and *in vivo* and the reported association between reduced expression of p21 and increased sensitivity to paclitaxel, we hypothesized that calcitriol would enhance the antitumor activity and apoptosis-promoting ability of paclitaxel.

MATERIALS AND METHODS

Tumor Cells and Model Systems. SCCVII/SF is a moderately well-differentiated SCC derived from a spontaneously arising tumor of the C3H mouse (20). SCCVII/SF cells were obtained from K. Fu (University of San Francisco, San Francisco, CA). They were transplanted in 6–10-week-old female C3H/HeJ mice (obtained from The Jackson Laboratory, Bar Harbor, ME). For *in vitro* studies, cells were grown in RPMI 1640 containing penicillin-streptomycin and 15% FCS (HyClone Laboratories, Inc., Logan, UT) at 37°C in a humidified atmosphere containing 5% CO_2. *In vivo*, SCCs were routinely produced by s.c. inoculation of 5×10^5 log-phase tissue culture cells in the right flank of each mouse. Studies were initiated ~9 days later when tumors were palpable.

The human prostatic adenocarcinoma cell line PC-3 was obtained from the American Type Culture Collection (Manassas, VA). For *in vitro* studies, cells were grown in F-12K medium containing penicillin-streptomycin and 10% FCS and 2 mM L-glutamine at 37°C in a humidified atmosphere containing 5% CO_2. *In vivo*, adenocarcinomas were routinely produced by s.c. inoculation of 2×10^6 log-phase tissue culture cells mixed 1:1 with Matrigel (Becton Dickinson, Bedford, MA) in the right flank of each nude mouse (Taconic Farms, Germantown, NY). Studies were initiated when tumors were palpable. Animals were used in accordance with Institutional Animal Care and Use Committee guidelines.

Chemicals and Reagents. Calcitriol (Hoffmann-LaRoche, Nutley, NJ) was reconstituted in 100% ethanol and stored protected from light under a layer of nitrogen gas at −70°C. All handling of calcitriol was performed with indirect lighting. Paclitaxel (Taxol; Bristol-Myers Squibb, Princeton, NJ) was purchased as a 6 mg/ml solution in Cremophor EL and was diluted in tissue culture medium or sterile saline just prior to use. The antibodies used in these studies were monoclonal mouse anti-PARP (Enzyme Systems, Livermore, CA), monoclonal mouse antihuman Bcl-2 (Dako, Carpinteria, CA), and polyclonal rabbit anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA). Antirabbit and antimouse horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Life Sciences (Arlington Heights, IL) and Promega Corp. (Madison, WI), respectively. Actin was detected using the actin (Ab-1) kit from Oncogene Research Products (Boston, MA).

***In Vitro* Clonogenic Tumor Cell Survival Assay.** Tumor cells were incubated for 24 h in T25 flasks (Corning Costar Corp., Cambridge, MA) with or without calcitriol. Cells were then either left untreated or were treated with various concentrations of paclitaxel for an additional 24 h. The cells were then harvested and counted, and a fixed number of cells were replated into six-well tissue culture plates (Corning Costar). After a 7-day incubation at 37°C in a humidified atmosphere containing 5% CO_2, cell monolayers were washed with saline, fixed with 100% methanol, and stained with 10% Giemsa. Colonies, defined as being >50 cells, were counted with the use of a light microscope. The surviving fraction is defined as follows: Surviving fraction = [fraction of viable cells recovered × (cloning efficiency of treated cells/cloning efficiency of untreated, control cells)].

MTT Assay and Drug Interaction Analysis. SCC cells were plated at 1.5×10^3 cells/well into 96-well tissue culture plates (Corning Glass Inc., Corning, NY) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. After a 24-h recovery, cells were either untreated or treated for 24 h with varying doses of calcitriol. Subsequently, cells received no further treatment or were treated for 24 h with varying doses of paclitaxel. Plates were harvested by staining with 0.5% MTT, and the absorbance was read with an ELISA reader (model EL-340; Bio-Tek Instruments, Winooski, VT) at 460 nM. Drug interactions were quantitated by median-dose effect analysis (21), and combination index values were derived using CalcuSyn software (Biosoft, Ferguson, MO), as described previously (22). CI values of <1, =1, and >1 indicate synergism, additivity, and antagonism between the drugs, respectively.

***In Vivo* Excision Clonogenic Assay.** Mice with 9-day SCCs (three to five animals/group) were treated with saline or 2.5 μg of calcitriol each day for 3 days. On day 3, mice also received varying doses of paclitaxel. Twenty-four h after the last injection, the animals were sacrificed, and their tumors were excised. Aliquots of minced tumor were enzymatically dissociated for 60 min at room temperature with a mixture of type I collagenase, DNase, and EDTA. For each treatment group, a fixed number of viable tumor cells, as determined by trypan blue staining, were then plated in six-well tissue culture plates. After incubation for 7 days, colonies were counted, and the surviving fraction was calculated using the equation: Surviving fraction = (the cloning efficiency of treated cells/cloning efficiency of untreated, control cells). The surviving fraction per gram of tumor is defined as the number of clonogenic tumor cells per gram of treated tumor divided by the number of clonogenic tumor cells per gram of control, untreated tumor.

Tumor Growth Inhibition. To examine the *in vivo* antitumor activity of calcitriol, paclitaxel, or the combination of calcitriol with paclitaxel, treatment was initiated on animals bearing palpable SCC or PC-3 tumors. Animals were treated for 3 days with single, daily i.p. injections of saline or calcitriol. On day 3, animals also received a single i.p. injection of paclitaxel. Tumor measurements were obtained using calipers prior to initiating treatment (initial tumor volume) and on the days indicated. Tumor volumes were calculated by the following formula: volume = (length × width^2)/2. For each tumor, fractional tumor volumes were calculated using the following formula: Fractional tumor volume = (volume on day measured)/(initial tumor volume).

Preparation of Cell Lysates and Western Blot Analysis. PC-3 cells were seeded into T75 flasks at densities of 1×10^4 to 2×10^4 cells/ml. Forty-eight h later, the medium was replaced. Treatments were done by adding concentrated drug

stocks directly to the culture media. At various times, cells that detached from the tissue culture plate were harvested by collecting the culture medium. After removing these cells, adherent cells were scraped up into PBS. Detached and adherent cells were maintained as separate populations. Cells were collected by centrifugation and washed once in PBS, and the resulting pellets were stored at −70°C. Protein extracts were prepared by resuspending cell pellets in lysis buffer [1% Triton X-100, 0.1% SDS, 50 mM Tris, and 150 mM NaCl, containing 1× protease inhibitor cocktail (PharMingen)] for 30 min on ice. Lysates were transferred to 1.5-ml Eppendorf tubes and clarified by centrifugation at 13,000 rpm for 10 min at 4°C. Proteins were quantitated in duplicate using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's directions. Protein lysates were stored at −70°C until use.

Proteins were resolved on SDS-polyacrylamide gels under denaturing conditions and then electrophoretically transferred to PVDF membranes (NEN Life Science Products, Boston, MA) overnight at 4°C. At room temperature, membranes were blocked for a minimum of 1 h in a 5% w/v solution of nonfat milk in TBST (10 mM Tris, pH 7.6, 150 mM NaCl, and 0.05% Tween 20) and then incubated for 1 h with primary antibody. The blots were washed three times in TBST and subsequently incubated with secondary antibody conjugated with horseradish peroxidase for 1 h. The blots were again washed, and the proteins were detected using Renaissance Western blot chemiluminescence reagents (NEN Life Science Products).

RESULTS

Calcitriol Increases Paclitaxel Antitumor Activity *in Vitro*. To examine the antitumor activity of calcitriol and paclitaxel alone, or in combination, murine SCC cells were used in an *in vitro* clonogenic assay. SCC cells were: (*a*) treated with paclitaxel or calcitriol alone; (*b*) pretreated for 24 h with calcitriol and then treated with paclitaxel; or (*c*) treated simultaneously with both agents. Paclitaxel was not administered prior to calcitriol in these studies because pilot *in vivo* experiments demonstrated that this schedule was associated with greater toxicity (data not shown). As we reported previously, calcitriol inhibits clonogenic survival in SCC with an IC_{50} of 4 nM (Fig. 1; Ref. 7). Paclitaxel alone also inhibits SCC survival, with an IC_{50} of 23 nM. Significantly greater antitumor activity was achieved when calcitriol was combined with paclitaxel (Fig. 1). Calcitriol pretreatment potentiated paclitaxel activity to a greater extent than concurrent treatment, except at the highest dose of paclitaxel studied. These results demonstrate that antitumor activity in SCC is increased by combining calcitriol with paclitaxel, and the optimal schedule for administration is treatment with calcitriol, followed by paclitaxel.

Similarly, we examined whether the combination of calcitriol and paclitaxel was effective in inhibiting the growth of human prostatic adenocarcinoma (PC-3) cells. PC-3 cells were treated *in vitro* for 24 h with or without calcitriol and received no further treatment or were treated for an additional 24 h with varying concentrations of paclitaxel. As shown in Fig. 2, calcitriol alone had detectable antiproliferative activity in these cells, with an IC_{50} of 5 μM. Paclitaxel, when used as a single agent, reduced PC-3 clonogenic survival in a concentration-dependent manner. Significantly greater growth inhibition was achieved by pretreating the cells with calcitriol at each of the paclitaxel concentrations tested. Therefore, the combination of calcitriol plus paclitaxel displays increased antiproliferative activity in murine SCC and human prostatic adenocarcinoma, indicating that the effects are not cell type specific.

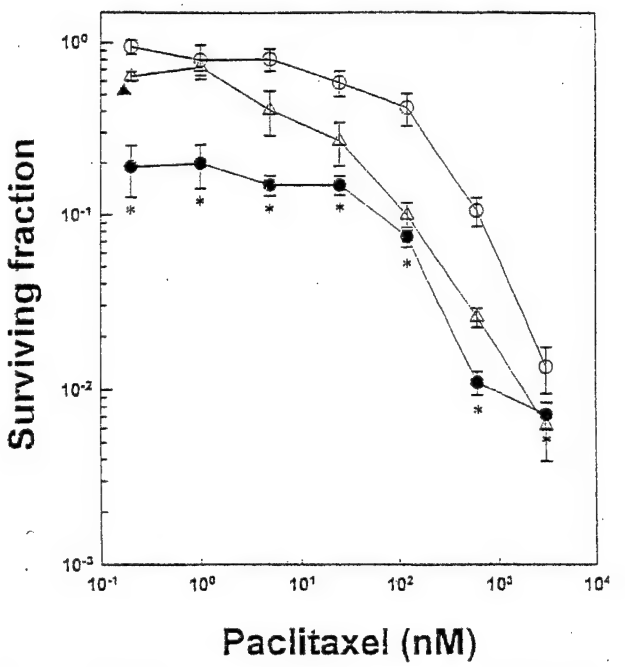

Fig. 1 Dose-response curves of SCC cells treated *in vitro* with calcitriol alone (▲), with varying doses of paclitaxel alone (○), pretreated with calcitriol followed by paclitaxel (●), or treated simultaneously with calcitriol plus paclitaxel (△), as measured by growth inhibition in the 7-day *in vitro* clonogenic assay. In pretreated cells, calcitriol was added 24 h prior to paclitaxel. Cells were exposed to paclitaxel for a total of 24 h. Calcitriol was used at a concentration of 4 nM. Each *point* represents the mean surviving fraction as determined by counting triplicate wells; *bars*, 95% confidence intervals. Note that the symbol for the calcitriol-alone treatment group is arbitrarily placed with respect to the *X axis*. Values for pretreatment with calcitriol followed by paclitaxel are significantly different from those obtained for paclitaxel alone; *, $P \leq 0.01$.

The Interaction between Calcitriol and Paclitaxel Is Synergistic *in Vitro*. Median-dose effect analysis (21) was used to evaluate the nature of the interaction between calcitriol and paclitaxel. SCC cells were treated with calcitriol or paclitaxel alone or were treated using the optimally defined sequence of calcitriol, followed by paclitaxel. Total drug exposures were fixed at 48 h for calcitriol and 24 h for paclitaxel. Dose-effect data were used to derive a combination index, as described previously (22). As shown in Fig. 3, CIs <1.0 were obtained for all combinations of calcitriol and paclitaxel examined, indicating that the interaction between the two drugs is synergistic.

Calcitriol Increases Paclitaxel Antitumor Activity *in Vivo*. To evaluate whether the combination of calcitriol and paclitaxel has greater *in vivo* antitumor activity compared with either agent alone, the excision clonogenic assay was used. As shown in Fig. 4*A*, the combination of calcitriol plus paclitaxel resulted in a significantly greater decrease in surviving fraction

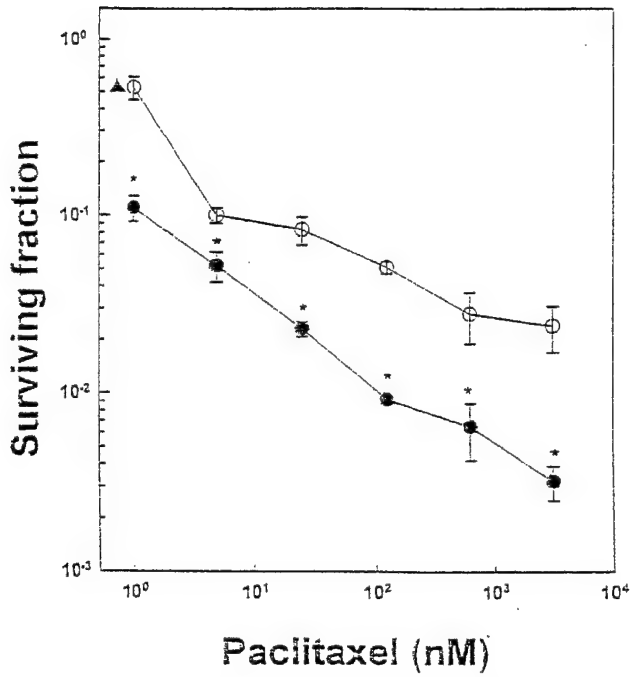

Fig. 2 Dose-response curves of PC-3 cells treated *in vitro* with calcitriol alone (▲), with varying doses of paclitaxel alone (○), or pretreated with calcitriol for 24 h followed by paclitaxel (●) as measured by growth inhibition in the 7-day *in vitro* clonogenic assay. Cells were exposed to paclitaxel for 24 h. Calcitriol was used at a concentration of 5 μM. Each *point* represents the mean surviving fraction as determined by counting triplicate wells; *bars*, 95% confidence intervals. Note that the symbol for the calcitriol-alone treatment group is arbitrarily placed with respect to the *X axis*. Values for pretreatment with calcitriol followed by paclitaxel are significantly different from those obtained for paclitaxel alone; *, $P < 0.0004$.

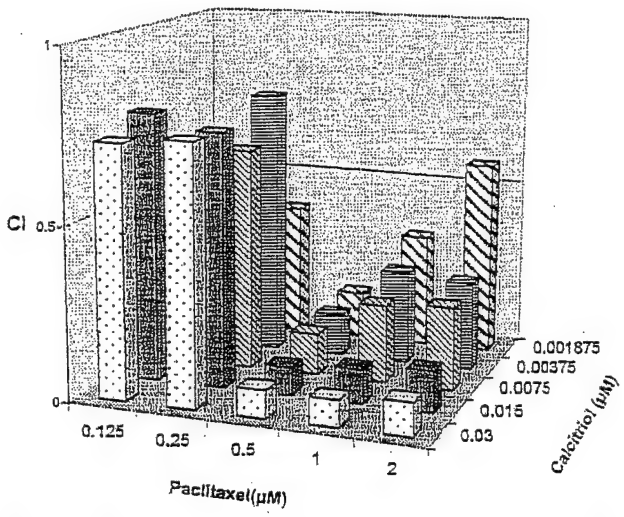

Fig. 3 Assessment of the interaction between calcitriol and paclitaxel in SCC. Cells were plated at 1.5×10^3 cells/well into 96-well tissue culture plates and allowed to recover for 24 h. Cells were then either untreated or pretreated for 24 h with the indicated doses of calcitriol. Subsequently, cells received either no further treatment or were treated for 24 h with the indicated doses of paclitaxel. Plates were harvested by staining with MTT, and the absorbance was read at 460 nM. The dose-effect data obtained for each drug alone and in combination were used to calculate CI values as described previously (22).

as compared with paclitaxel or calcitriol alone. Thus, greater *in vivo* antitumor activity is achieved in SCC by pretreatment with calcitriol followed by paclitaxel.

To determine whether an increase in clonogenic cell kill was associated with inhibition of tumor growth, SCC tumor-bearing mice were treated with saline, calcitriol or paclitaxel alone, or calcitriol in combination with paclitaxel. We used a schedule of daily × 3 doses of calcitriol with paclitaxel administered on day 3. This calcitriol dosing regimen was reported previously to maximize antitumor efficacy while minimizing toxicity or hypercalcemia (13). In SCC, paclitaxel had no significant activity when used as a single-agent therapy, and calcitriol alone exhibited cytostatic activity (Fig. 4*B*). In contrast, the combination of calcitriol with paclitaxel resulted in significant tumor regression (Fig. 4*B*).

To determine whether calcitriol plus paclitaxel combination therapy also displays increased antiproliferative activity *in vivo* in PC-3, tumor-bearing mice were treated with saline, calcitriol or paclitaxel alone, or calcitriol in combination with paclitaxel using the dosing schedule described for SCC. As shown in Fig. 5*A*, neither paclitaxel nor calcitriol had significant activity when used as a single-agent therapy in PC-3. However, significant antitumor activity was observed when PC-3 tumor-bearing mice were treated with calcitriol followed by paclitaxel (Fig. 5*A*). In this model, nearly complete inhibition of tumor growth was maintained for >2 weeks.

After day 24, PC-3 tumors in animals treated with calcitriol plus paclitaxel achieved a growth rate comparable with controls as determined by the slope (m) of the growth curve (0.107 *versus* 0.102). Retreatment of these animals with calcitriol plus paclitaxel on days 29–31 resulted in a decrease in the rate of tumor growth, as evidenced by a change in the slope of the growth curve for days 32–38 (m, 0.052; Fig. 5*B*). These data indicate that previously treated tumors remain responsive to calcitriol plus paclitaxel and that prolonged antitumor activity may be achieved by repeated cycles of therapy.

Calcitriol Decreases p21 Expression and Accelerates Paclitaxel-induced Apoptosis. Given the findings that loss of p21 sensitizes MCF-7 and HCT116 cells to paclitaxel (17, 18) and calcitriol decreases p21 expression in SCC (14), we hypothesized that calcitriol enhances paclitaxel antitumor activity via its effects on p21. To test whether calcitriol treatment decreases p21 expression in PC-3 as it does in SCC, cells were treated *in vitro* with ethanol vehicle control or calcitriol. At various times, whole cell lysates were prepared and analyzed for p21 expression by Western blot. As shown in Fig. 6, calcitriol treatment resulted in a 60% decrease in p21 expression in PC-3 cells after 72 h and an 80% decrease in expression after 96 h.

To determine whether PC-3 cells with reduced p21 expression are more sensitive to paclitaxel than cells with baseline p21 expression, cells were pretreated for 72 h with ethanol solvent control or calcitriol as indicated in Table 1. Cells subsequently received no further treatment or were treated with paclitaxel alone or calcitriol plus paclitaxel. Viable cells were counted 24 h after paclitaxel addition. Coadministration of calcitriol plus pa-

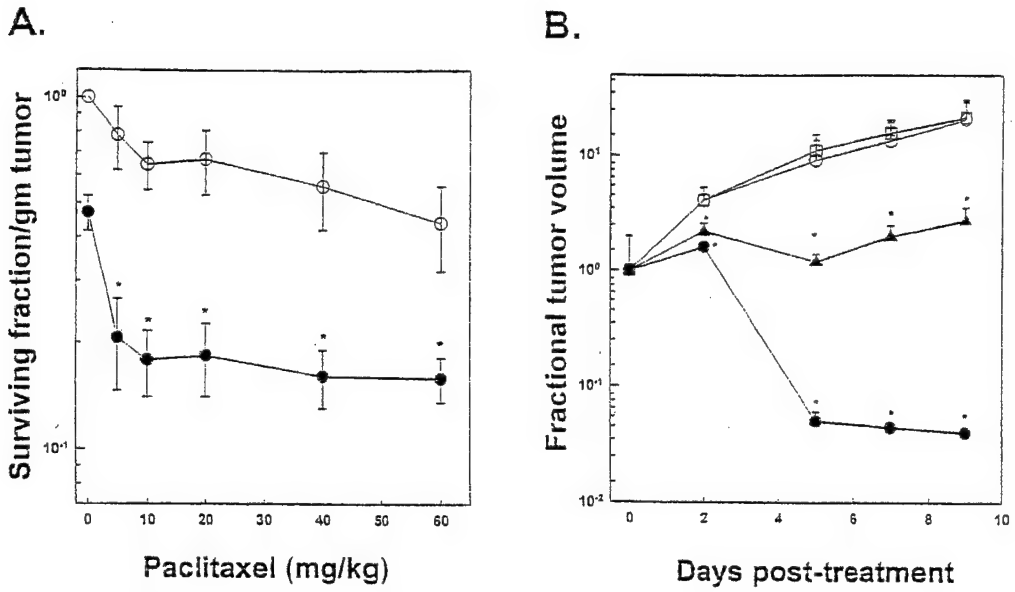

Fig. 4 Calcitriol increases paclitaxel antitumor activity in SCC *in vivo*. *A*, SCC tumor-bearing mice were treated with saline (○) or 2.5 μg of calcitriol each day for 3 days (●). On the third day, mice also received varying i.p. doses of paclitaxel (0–60 mg/kg). Twenty-four h later, tumors were harvested, dissociated, and plated in the excision clonogenic assay. Colonies were enumerated after 7 days. Each *point* represents the mean surviving fraction for total clonogenic cells/gram of tumor (three to five mice per treatment group). *, values for treatment with calcitriol followed by paclitaxel are significantly different from those obtained for paclitaxel alone; $P < 0.01$. *B*, C3H mice bearing palpable, s.c. SCC tumors were treated with either saline (□), 1.25 μg of calcitriol daily for 3 days (▲), 20 mg/kg paclitaxel on day 3 (○), or the combination of 1.25 μg of calcitriol daily for 3 days plus 20 mg/kg paclitaxel on day 3 (●). Both agents were administered i.p. Tumor measurements were obtained on the days indicated, and fractional tumor volumes were calculated as described in "Materials and Methods." Data points represent the mean fractional tumor volume for five animals/group; *bars*, SD. Values significantly different from no treatment are shown. *, $P < 0.01$.

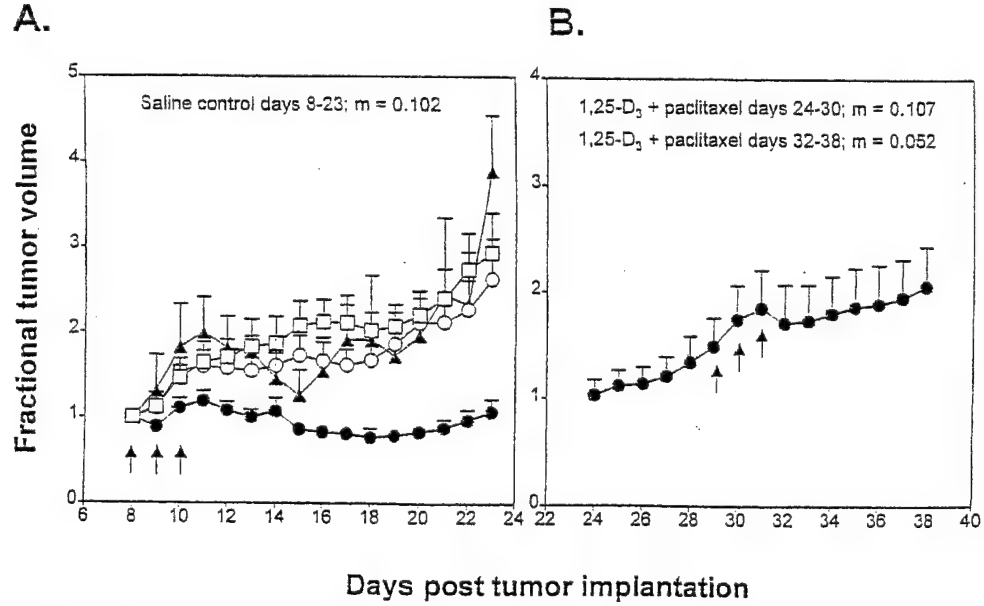

Fig. 5 Calcitriol increases paclitaxel antitumor activity in PC-3 *in vivo*. *A*, nude mice bearing palpable, s.c. PC-3 tumors were treated with either saline (□), 0.75 μg of calcitriol daily for 3 days (▲), 10 mg/kg paclitaxel on day 3 (○), or the combination of 0.75 μg of calcitriol daily for 3 days plus 10 mg/kg paclitaxel on day 3 (●). Both agents were administered i.p. Fractional tumor volumes were calculated as described in the legend for Fig. 4*B*. Data *points* represent the mean fractional tumor volume for five animals/group; *bars*, SD. *Arrows*, treatment days. *B*, animals treated previously with calcitriol plus paclitaxel on days 8–10 were treated with a second cycle of therapy on days 29–31. *m*, the slope of the growth curve derived by linear regression of tumor volume data obtained on the days indicated.

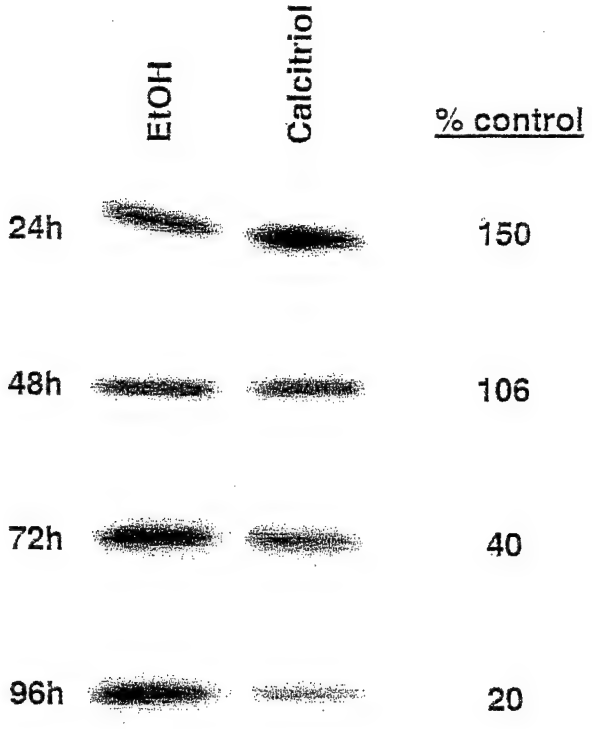

Fig. 6 Calcitriol modulates expression of the cdk inhibitor p21 in PC-3 cells. Whole cell lysates were prepared from subconfluent PC-3 cells treated *in vitro* with ethanol solvent control or 5 μM calcitriol for 24–96 h. Proteins were resolved on SDS-polyacrylamide gels under denaturing conditions, transferred to PVDF membrane, and analyzed by Western blot using polyclonal anti-p21 antibodies. Protein expression levels were quantitated by densitometry and are expressed as a percentage of ethanol control at each time point. A representative experiment is shown. *EtOH*, ethanol.

clitaxel resulted in a greater reduction in cell number than treatment with either agent alone (Table 1). However, the effect of the combination was only additive. In contrast, a greater than additive reduction in cell number was obtained when cells were pretreated with calcitriol followed by paclitaxel. Thus, a calcitriol pretreatment that reduces p21 expression further enhances paclitaxel cytotoxicity in PC-3.

Paclitaxel-induced apoptosis in PC-3 is associated with phosphorylation of Bcl-2 (23), which inactivates the apoptotic suppressor function of this protein (23–25). Therefore, to determine whether PC-3 cells with reduced p21 expression show enhanced paclitaxel activity at the molecular level, we examined whether calcitriol pretreatment increased or accelerated paclitaxel-induced changes in Bcl-2 expression and apoptosis. As outlined in Table 1, PC-3 cells were either treated with ethanol control or pretreated with 5 μM calcitriol for 72 h, a time sufficient for p21 down-modulation. Subsequently, cells received no further treatment, were treated with paclitaxel alone, or were treated with calcitriol plus paclitaxel for varying lengths of time. Inspection of treated cells revealed two morphologically distinct populations, one cell population remained adherent after treatment whereas a second population detached from the culture dishes. These populations were analyzed separately.

At 24 h, paclitaxel induced Bcl-2 phosphorylation in both the adherent and detached cell populations (Fig. 7). Whereas unphosphorylated Bcl-2 was most abundant in the paclitaxel-treated adherent cells, only the phosphorylated forms of Bcl-2 were detected in the detached cells. Phosphorylated Bcl-2 species were still detected in both cell populations after 48 h of treatment but were virtually absent by 72 h. Calcitriol had little effect on Bcl-2, and neither pretreatment nor concurrent treatment with calcitriol altered the effects of paclitaxel on Bcl-2 expression/phosphorylation.

Apoptosis, as measured by loss of full-length PARP, first became evident in the detached cells after 48 h of treatment (Fig. 7). At this time, paclitaxel treatment resulted in a 50% reduction in PARP. Although calcitriol itself did not induce apoptosis, it enhanced the effects of paclitaxel such that cells treated with calcitriol prior to or in combination with paclitaxel displayed a 78% reduction in PARP. By 72 h, PARP was no longer detected in the cells that detached after paclitaxel administration, and addition of calcitriol had no further discernible effect. Calcitriol did not alter the effects of paclitaxel on PARP expression in the adherent cell population at any of the times examined. The data obtained at 48 h indicate that calcitriol can accelerate paclitaxel-induced apoptosis in a subset of PC-3 cells in a schedule-independent manner.

DISCUSSION

On the basis of epidemiological findings, Schwartz and Hulka (26) proposed a protective role for calcitriol in prostate cancer. Subsequently, the antiproliferative activity of calcitriol on prostatic adenocarcinoma cell lines *in vitro* (1, 2, 11) and *in vivo* (1, 27) was demonstrated. Antiproliferative effects of calcitriol were also observed in a pilot clinical trial in which, in a small set of patients with early, recurrent prostate cancer, calcitriol decreased the rate of PSA rise, resulting in an increase in PSA doubling times (28). PSA responses have also been observed in our ongoing Phase II trial of calcitriol plus dexamethasone in hormone-refractory prostate cancer (29). In this trial, 8–12 μg of calcitriol was given p.o. Monday, Tuesday, and Wednesday each week with 4 mg of dexamethasone given Sunday, Monday, Tuesday, and Wednesday. Among evaluable patients, 21% experienced a greater than 50% decrease in PSA, and 79% experienced a decrease in PSA velocity. In a further effort to develop new calcitriol-based therapies for advanced malignancy, we investigated the effect of combining calcitriol with cytotoxic agents.

Preclinically, we demonstrated that there is an increase in antitumor activity in prostatic adenocarcinoma using calcitriol in combination with paclitaxel *in vitro* and *in vivo* as measured in clonogenic assays and tumor growth inhibition studies. On the basis of these findings, we propose that calcitriol plus paclitaxel combination therapy may have utility in the treatment of patients with prostate cancer.

The clinical use of calcitriol may be restricted by its dose-limiting toxicity, hypercalcemia. However, a variety of calcitriol analogues, including ILX-23–7553 and EB1089, have been described that possess antiproliferative activity *in vivo* without inducing hypercalcemia (7, 30). It has been shown recently that EB1089, when combined with paclitaxel, inhibits the growth of

Table 1 Effect of *in vitro* treatment on recovery of viable PC3 cells

PC3 cells (2×10^5) were seeded and allowed to recover for 48 h. Cells were then treated for 72 h with either ethanol solvent control or calcitriol, as indicated. For the second treatment, concentrated stocks of paclitaxel alone or calcitriol plus paclitaxel were added directly to the cultures. The final concentrations of calcitriol and paclitaxel were 5 μM and 100 nM, respectively. Twenty-four h after the second treatment, cells were harvested, and viable cells were counted using trypan blue. The percentage of reduction in viable cell number was calculated using the equation: $1 - (\text{cell number}_{\text{treated}}/\text{cell number}_{\text{EtOH control}}) \times 100$.

Group	First tx[a] (72 h)	Second tx (24 h)	Cell no. ($\times 10^6$)	% reduction (in cell no.)
Ethanol	Ethanol	None	3.37	0
Calcitriol	Calcitriol	None	2.92	13.3
Ptx	Ethanol	Ptx	3.17	5.9
Calcitriol + Ptx	Ethanol	Calcitriol + Ptx	2.72	19.3
Calcitriol/Ptx	Calcitriol	Ptx	2.05	39.3

[a] tx, treatment; Ptx, paclitaxel.

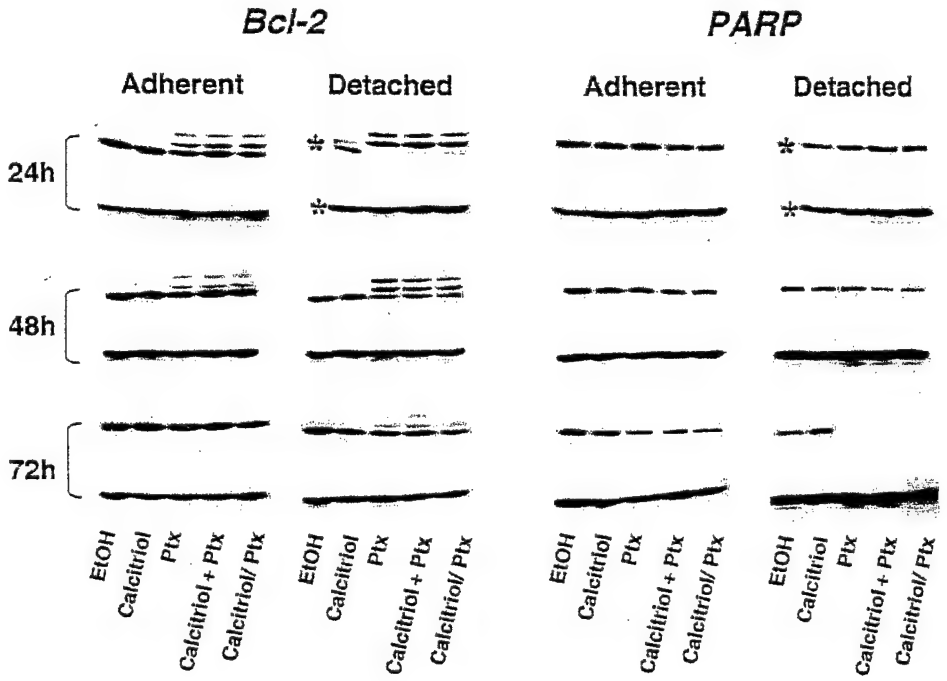

Fig. 7 Paclitaxel modulates expression of apoptosis-associated proteins Bcl-2 and PARP in PC-3 cells. Whole cell lysates were prepared from subconfluent PC-3 cells treated *in vitro* with ethanol, 5 μM calcitriol, 100 nM paclitaxel, or 5 μM calcitriol concurrent with (*Calcitriol + Ptx*) or prior to (*Calcitriol/Ptx*) 100 nM paclitaxel. Calcitriol pretreatment was for 72 h. Time *points* are hours after paclitaxel addition. Proteins were resolved on SDS-polyacrylamide gels under denaturing conditions, transferred to PVDF membrane, and analyzed by Western blot using antibodies raised against Bcl-2 or PARP. To verify equal protein loading, the Bcl-2 and PARP blots were reprobed for actin. For each time point, the Bcl-2 or PARP blot is shown directly above its corresponding actin blot. *, no floating cells were obtained. A representative experiment is shown.

MCF-7 breast cancer cells *in vivo* (31). Furthermore, we observed that paclitaxel appears to attenuate calcitriol-mediated hypercalcemia in preclinical models (data not shown). Agents that disrupt or stabilize microtubules can inhibit calcium transport, which may account for this activity (32). Thus, paclitaxel and either calcitriol or analogues may be a safe and effective combination in the treatment of human cancer.

We have further demonstrated that calcitriol enhances paclitaxel antiproliferative activity *in vitro* and *in vivo* in the murine SCC model, SCCVII/SF. A previous report indicates that these cells are relatively resistant to paclitaxel *in vivo* at a concentration of 40 mg/kg (33). We found that although paclitaxel (20 mg/kg) has little activity when administered to tumor-bearing mice as a single agent, pretreatment with calcitriol yields substantial antitumor activity (Fig. 4*B*). These data suggest that calcitriol and paclitaxel combination therapy may be useful, even in the treatment of tumors that are paclitaxel insensitive.

Paclitaxel cytotoxicity is increased in MCF-7 breast cancer cells and HCT116 colon cancer cells when p21 expression is specifically perturbed (17, 18). Because calcitriol treatment reduces p21 expression in the SCC model (14) and in PC-3 cells (Fig. 6), we hypothesized that calcitriol might enhance the antitumor activity of paclitaxel via its effect on p21. To test this, we examined whether paclitaxel effects were enhanced in cells pretreated with calcitriol (p21 low) as compared with cells receiving concurrent calcitriol (baseline p21) and paclitaxel.

Paclitaxel effects were measured by following changes in viable cell number (Table 1) and molecular markers (Fig. 7). In the cell recovery studies, we observed a schedule dependence such that the greatest reduction in cell number occurs when cells are pretreated with calcitriol for 72 h followed by a 24-h treatment with paclitaxel. Interestingly, the reduction in cell number cannot be accounted for by an increase in apoptosis because no PARP cleavage was detected 24 h after paclitaxel addition (Fig. 7). However, a different observation is made 48 h

after paclitaxel addition. At this time, calcitriol alone does not induce apoptosis but does enhance the level of PARP cleavage observed in the detached cell population in the presence of paclitaxel. This enhancement is schedule independent, with similar effects observed in paclitaxel-treated cells regardless of whether they were pretreated or concurrently treated with calcitriol.

On the basis of these data, we propose a model in which the "early" (within 24 h) enhancement of paclitaxel cytotoxicity by calcitriol is schedule dependent and is not attributed to acceleration of paclitaxel-induced apoptosis. The schedule dependence may reflect the time required for calcitriol treatment to decrease p21 expression. The "delayed" (at 48 h) enhancement of paclitaxel activity by calcitriol is schedule independent and associated with acceleration of apoptosis in a subset of PC-3 cells. Recent work from our laboratory demonstrates that calcitriol inhibits specific survival signals in cells that detach during treatment.[4] Such inhibition may render these cells more susceptible to the proapoptotic signals generated by paclitaxel. The ability of calcitriol to reduce cell survival signals may also explain how it can enhance the antitumor activity of mechanistically diverse cytotoxic agents, such as cisplatin (13) and paclitaxel. Studies to address these and related issues are in progress.

Paclitaxel-mediated apoptosis in LNCaP and PC-3 prostate cancer cells has been associated with Bcl-2 phosphorylation and inactivation (23) and/or down-modulation of the related apoptotic suppressor, Bcl-X_L (34). Consistent with these results, we found that within 24 h, paclitaxel treatment resulted in phosphorylation of the apoptotic suppressor protein, Bcl-2. Loss or inactivation of Bcl-2 in prostate cancer cells after paclitaxel administration has been proposed to promote cell death by shifting the intracellular balance of death regulators in favor of proapoptotic molecules such as Bax (23). In our studies, paclitaxel-mediated changes in the intracellular levels of Bcl-2 temporally precede the loss of full-length PARP, suggesting that they may initiate the apoptotic program.

Wang *et al.* (35) demonstrated recently that calcitriol pretreatment increases paclitaxel induction of cell death and paclitaxel antitumor activity *in vitro* in MCF-7 breast cancer cells. However, in contrast to our findings, calcitriol modestly increased the effect of paclitaxel on Bcl-2 phosphorylation. Comparison of these two studies reveals that although a 24-h exposure to 100 nM paclitaxel results in minimal Bcl-2 phosphorylation in MCF-7, this exposure results in strong induction of Bcl-2 phosphorylation in PC-3. This strong induction may have prohibited the detection of a subtle effect of calcitriol on paclitaxel-mediated Bcl-2 phosphorylation in PC-3.

In summary, our data demonstrate that calcitriol enhances paclitaxel antitumor activity in PC-3 and SCC cells *in vitro* and *in vivo* and indicate that novel calcitriol/paclitaxel-based combination therapies may have significant clinical utility in the treatment of a variety of solid tumors.

[4] T. F. McGuire, D. L. Trump, and C. S. Johnson. Vitamin D_3-induced apoptosis of murine squamous cell carcinoma cells: selective induction of caspase-dependent MEK cleavage and up-regulation of MEKK1. submitted for publication.

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Vitamin D_3-induced apoptosis of murine squamous cell carcinoma cells: Selective induction of caspase-dependent MEK cleavage and up-regulation of MEKK-1.*

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Running Title: D_3 induces MEK cleavage, MEKK-1 up-regulation, and apoptosis

Vitamin D_3 inhibits cell growth and induces apoptosis in several human cancer lines in vitro and in vivo. However, little is known about the molecular events involved in vitamin D_3-induced apoptosis. Here, we demonstrate that the growth-promoting/pro-survival signaling molecule mitogen-activated protein kinase kinase (MEK) is cleaved in a caspase-dependent manner in murine squamous cell carcinoma (SCC) cells induced to undergo apoptosis by treatment with vitamin D_3. Cleavage resulted in nearly complete loss of full-length MEK and Erk1/2 phosphorylation. Erk1/2 expression was affected only slightly. The phosphorylation and expression of Akt, a kinase regulating a second cell survival pathway, was also inhibited after treatment with vitamin D_3. However, the pro-apoptotic signaling molecule MEKK-1 was up-regulated in both apoptotic and non-apoptotic cells with greater induction and partial N-terminal proteolysis of MEKK-1 observed in apoptotic cells. In contrast to vitamin D_3, cisplatin and etoposide down-regulated Akt levels only modestly, did not promote significant loss of MEK expression, and did not up-regulate MEKK-1. We propose that vitamin D_3 induces apoptosis in SCC cells by a unique mechanism involving selective caspase-dependent MEK cleavage and up-regulation of MEKK-1. Additional evidence is provided that vitamin D_3-induced apoptosis may be mediated via p38 MAPK.

1,25-dihydroxycholecalciferol (vitamin D_3)[1] is the active metabolite of vitamin D and exhibits anti-proliferative and differentiation-promoting activities in vitro toward a number of malignant cell types, including breast cancer cells (1,2), prostate cancer cells (3,4), colorectal adenoma and carcinoma cells (5), melanoma cells (6), and mouse myeloid leukemia (7-9). In addition, vitamin D_3 inhibits prostate adenocarcinoma growth and metastasis in the Dunning rat prostate model system (10) and displays anti-neoplastic activity in a variety of human xenograft tumor model systems (11,12). Several vitamin D_3 analogues have also been developed and display similar activities in vitro (5,13-15) and in vivo (16-18). Furthermore, we have demonstrated that vitamin D_3 inhibits the proliferation of squamous cell carcinoma (SCC) cells in vitro as well as the growth of newly transplanted or established SCC tumors in vivo (19,20). Hence, the accumulated evidence clearly establishes the anti-proliferative activity of vitamin D_3 (and its analogues) against several cancer cell types and supports the examination of their potential usefulness as anti-cancer agents (reviewed in refs. 12 and 21).

On the cellular level, vitamin D_3 has been shown to exert its anti-proliferative effects by inhibiting cell cycle progression and/or by promotion of programmed cell death (apoptosis). G1 arrest induced by vitamin D_3 or vitamin D_3 analogues has been attributed to a number of molecular changes, including induction of the cyclin-dependent kinase (cdk) inhibitors p21Waf1 and p27Kip1, inhibition of cdk2 activity, hypophosphorylation of the retinoblastoma protein (Rb), and suppression of E2F activation (4,18,20,22-24). Vitamin D_3-induced apoptosis, while being demonstrated for several cancer cell lines derived from various tissues, including breast (2,3,17,25,26), prostate (3,27), colon (5,13), and skin (6) in vitro, as well as breast cancer cells in vivo

(28), is less well defined. Levels of the anti-apoptotic molecule Bcl-2 have been shown to be reduced after vitamin D_3 treatment in some cell lines, including MCF-7 human breast cancer cells (17,26,28) and LNCaP human prostate cancer cells (27). Furthermore, vitamin D_3-induced apoptosis in these cell lines is blocked by over-expression of Bcl-2 (27,29). While these results suggest a possible role for Bcl-2 down-regulation in vitamin D_3-induced apoptosis, it is not clear whether reduction of Bcl-2 expression is sufficient of itself to induce apoptosis. In addition, vitamin D_3-induced apoptosis of colon cancer cells is not dependent on Bcl-2 down-regulation, but is more tightly associated with up-regulation of the pro-apoptotic molecule Bak (5). Another body of work suggests that vitamin D_3 induces insulin-like growth factor binding proteins (IGFBPs) which bind to IGF-1, preventing it from stimulating the pro-survival IGF-1 receptor, and thereby promote apoptosis (30-32). Finally, Mathiasen et al. (29) recently reported that sensitivity of MCF-7 cells to vitamin D_3-induced apoptosis does not depend on expression of a functional p53 tumor suppressor protein and does not involve the activation of known caspases. Thus far, the involvement of stress and survival signal transduction pathways in the mechanism of vitamin D_3-induced apoptosis has not been carefully examined.

The integration of multiple signals from numerous transduction pathways plays a critical role in regulating cell survival and execution of programmed cell death (reviewed in (33) and (34)). While activation of stress signals from the SEK1-JNK and the MKK3/6-p38 MAPK pathways can push the cell toward apoptosis (35-38), opposing signals generated from the MEK-Erk and PI-3 kinase-Akt pathways antagonize the death signals and mediate survival (39-45). When the integrated signals from these pathways

begin to favor apoptosis, caspases can become activated, resulting in the selective cleavage of a distinct set of proteins and greatly enhancing the potential toward commitment to apoptosis. While cleavage of target proteins by caspases typically results in their inactivation, exceptions to this include the caspases themselves as well as two stress-signaling proteins, MEKK-1 and PAK2 (46,47). MEKK-1 is a 196 kDa Ser/Thr kinase which, upon phosphorylation, becomes activated and transduces stress signals that lead to the activation of JNK and p38 MAPK (48), thereby promoting apoptosis. Upon treatment of cells with genotoxic agents (e.g., etoposide or UV-C irradiation), MEKK-1 initially undergoes phosphorylation-dependent activation and is subsequently cleaved by caspase-3, releasing the N-terminal regulatory domain and producing a constitutively active C-terminal kinase domain (49). Similarly, MEKK-1 cleavage is also observed in cells induced to undergo apoptosis by Fas ligation (50) and by detachment from the extracellular matrix (anoikis) (46). Interestingly, cells expressing a non-cleavable MEKK-1 mutant are impaired in their ability to undergo apoptosis after treatment with DNA-damaging agents (though mutant MEKK-1 is still phosphorylated) (49). Thus, MEKK-1 cleavage is not only a common biological event observed during apoptosis induced by a variety of agents, but appears to play a necessary role in the execution of the apoptotic program. Caspase-mediated cleavage of PAK2, a ser/thr kinase that regulates cytoskeletal changes in many cell types, also appears to play a role in the apoptotic process (47).

We have investigated the mechanism by which vitamin D_3 induces apoptosis in SCC cells, particularly with respect to its effects on survival and stress signaling pathways. We report here that vitamin D_3 induces the caspase-dependent cleavage of

MEK, not previously described, resulting in nearly complete loss of MEK expression and Erk1/2 signaling. Moreover, Akt signaling is potently inhibited in cells induced to undergo apoptosis by vitamin D_3. Furthermore, vitamin D_3 strongly induces MEKK-1 expression in cells prior to onset of apoptosis and subsequently promotes N-terminal proteolysis such that numerous MEKK-1 fragments are observed in the apoptotic cells. Finally, direct comparison of vitamin D_3 with genotoxic agents revealed that the molecular events described above, with the exception of Akt inhibition, were selective for vitamin D_3.

EXPERIMENTAL PROCEDURES

Culture and Treatment of SCC Cells. Murine squamous cell carcinoma (SCC) cells (either 3 x 10^5 or 6 x 10^5 cells/T-75 flask) were plated in RPMI 1640 medium supplemented with 12 % fetal bovine serum (FBS), unless otherwise noted (see Fig. 2). After 1 or 2 days, the cells were treated at sub-confluence with either vehicle (< 0.001 % ethanol, EtOH), 10 nM 1,25-dihydroxycholecalciferol (1,25-D_3), 1 μg/ml cisplatin, or 10 μM etoposide. After 2 days of treatment, medium in the flasks was gently swirled to suspend floating (apoptotic) cells, which were then harvested by centrifugation, and washed once with ice-cold PBS. Attached (non-apoptotic) cells were rinsed once with PBS and either scraped off the flask surface into PBS and placed in a separate tube (for Western blotting), or trypsinized, suspended in PBS, and combined with the floating cells (for YO-PRO-1 staining). For experiments assessing the role of caspases in vitamin D_3-induced MEK cleavage, cells were treated with either vehicle or 10 nM vitamin D_3 in the presence or the absence of either 20 μM Z-DEVD-FMK or 20 μM Z-VAD(OMe)-FMK and the cells harvested, as described above.

Western Blotting. Both attached and floating cell populations were then lysed in buffer containing the following: 50 mM Tris, pH 8.0, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, 1 mM benzamidine, 5 mM N-ethylmaleimide, 2 mM vanadate, 2 mM EGTA, 12 mM β-glycerol phosphate, 10 mM NaF, and 10 nM okadaic acid. Cell lysates were cleared by centrifugation at 14,000 rpm for 10 min at 4°C and

aliquots were incubated with an equivalent volume of 2X SDS-PAGE sample buffer for at least 4 min at 100°C. Equivalent amounts of protein from each sample were electrophoresed on either 7.5 % SDS-PAGE (for PARP and MEKK-1) or 12.5 % SDS-PAGE (for all other antibodies) and transferred to PVDF membranes. Membranes were blocked with 5 % nonfat dry milk in Tris-buffered saline containing 0.05 % Tween-20 (T-TBS) and then probed with either anti-PARP (PharMingen, 65196E), anti-phospho-MEK1/2 (New England BioLabs, 9121S), anti-MEK1/2 (New England BioLabs, 9122), anti-phospho-Erk1/2 (Santa Cruz, sc-7383), or anti-Erk1/2 (Upstate Biotechnology, 06-182), anti-phospho-Akt (New England BioLabs, 9271), anti-Akt (New England BioLabs, 9272) anti-MEKK-1 (Santa Cruz, sc-252), anti-SEK1 (Santa Cruz, sc-964), anti-phospho-p38 MAPK (New England BioLabs, 9211), anti-p38 MAPK (Santa Cruz, sc-535), anti-phospho-c-Jun (Santa Cruz, sc-822), and anti-actin (Oncogene, CP01) in 3 % nonfat dry milk in T-TBS. Anti-phospho-MEK, anti-phospho-Erk1/2, anti-phospho-Akt, and anti-phospho-p38 MAPK antibodies selectively detect MEK phosphorylated at serines-217/221, Erk1/2 phosphorylated at tyrosine-204, Akt phosphorylated at serine-473, and p38 MAPK phosphorylated at threonine-180 and tyrosine-182, respectively, sites of phosphorylation necessary for full activation of these kinases. The immunogen used to generate the anti-MEKK-1 antibody (sc-252) was a 22 amino acid peptide, the sequence of which corresponded to a region lying within the last 50 amino acids of the rat MEKK-1 C-terminus (identical in sequence to that region found in the mouse MEKK-1 C-terminus). Positive antibody reactions were visualized using an appropriate HRP-conjugated secondary antibody and enhanced chemiluminescence (ECL) (NEN) [for PARP immunoblotting, ECL SuperSignal (Pierce) was used].

It is important to note that, although both the anti-phospho-MEK and anti-MEK antibodies recognized both MEK isoenzymes (MEK1 and MEK2), these two proteins were typically not completely resolved under the electrophoresis conditions used for this work. Both proteins, however, appeared to behave in a similar manner in response to the various treatment conditions described above. Therefore, in this report, we have represented both of the isoenzymes together by denoting them as "MEK" in the text and in the figures.

YO-PRO-1 Staining. As described above, at the time of harvest, floating cells were first removed with the medium and placed in a conicle tube; attached cells were rinsed once, trypsinized, suspended in RPMI 1640 medium containing 12 % FBS, and combined with the floating cells. Cells were then pelleted, resuspended in PBS to a concentration of 1.0 – 1.5 x 10^6 cells/ml, and the percent of apoptotic cells measured using the Vybrant Apoptosis Assay Kit #4 (Molecular Probes). According to the manufacturer's instructions, 1.0 µl each of YO-PRO-1 dye and propidium iodide solutions were added to 1.0 ml aliquots of suspended cells, allowed to incubate for at least 20 min, and subsequently analyzed by flow cytometry.

RESULTS

Vitamin D_3 induces apoptosis in murine squamous cell carcinoma (SCC) cells. We have reported previously that vitamin D_3 predominantly induces murine SCC cells to undergo cytoplasmic spreading (flattening), and become growth arrested in G1 phase of the cell cycle (19,51). However, a significant population of cells is also observed detached and floating in the culture medium after vitamin D_3 treatment and possibly represents apoptotic cells. In order to determine whether this population of cells exhibits characteristics of apoptosis, SCC cells were treated with vitamin D_3 (10 nM) for 2 days and assessed for apoptosis by various criteria. As shown in Fig. 1A, vitamin D_3 induced a fraction of the cells to remain attached and to spread out while another fraction became detached and exhibited cellular condensation and membrane blebbing, characteristics of cells undergoing apoptosis. The cells were then harvested, stained with propidium iodide and YO-PRO-1, and subjected to flow cytometric analysis. YO-PRO-1 is a fluorescent dye taken up by cells in early and late apoptosis (as well as necrotic cells). Based on this type of analysis, vitamin D_3 increased the proportion of cells in early apoptosis (0.2 to 11 %) and the total number of cells in apoptosis/necrosis (14 to 40 %) (the average induction of total apoptotic/necrotic cells from four independent experiments was from 13 to 36 %, or 2.8-fold) (Fig. 1B). Although flow cytometry detected significant basal levels of apoptosis in vehicle-treated cultures, no apoptosis could be detected in these cultures using the other two methods employed [morphological changes and caspase activation (see below)]. Thus, it appears that the basal level of apoptosis in vehicle-treated cultures

is artifactual and probably due to the trypsinization procedure used to prepare the cells for FACS analysis (see Experimental Procedures). Finally, we assessed induction of apoptosis by vitamin D_3 on the molecular level by examining caspase activation (i.e., by measuring the extent of cleavage of the caspase substrate poly(ADP-ribose) polymerase, or PARP). After treating SCC cells as described above, the floating cells were removed from the attached cells and lysates of both cell populations were prepared (described in Experimental Procedures). The number of floating cells in the medium after vehicle treatment was low and it was not possible to collect sufficient amounts to process for immunoblot analysis. As shown in Fig. 1C (lanes 1-2), lysates of vehicle-treated (attached) cells, as well as the attached cell population of vitamin D_3-treated cultures, exhibited no PARP cleavage. However, lysates of floating cells from cultures treated with vitamin D_3 demonstrated complete cleavage of PARP (116 kDa) to an 85 kDa fragment (Fig. 1C, lane 3). Taken together, these results indicate that vitamin D_3 induces programmed cell death in SCC cells. The fact that PARP cleavage was exclusively observed in lysates from vitamin D_3-treated, non-attached cells and that this population is easily isolated from the attached cell population affords the use of this model for investigating at the molecular level the role of signaling pathways in vitamin D_3-induced apoptosis.

Vitamin D_3 induces caspase-dependent cleavage of MEK in apoptotic cells. The Ras-Raf1-MEK-Erk signaling pathway transduces signals from growth factor receptors to the nucleus, which can lead not only to mitogenesis and differentiation but also to survival (34). Some investigators studying vitamin D_3-induced apoptosis have reported

that culturing cells under serum-free conditions enhances the apoptosis-promoting activity of vitamin D_3 (31). Thus, growth factor signaling may attenuate the apoptotic signal induced by vitamin D_3, or, conversely, vitamin D_3 may attenuate growth factor signaling as part of its mechanism to promote programmed cell death. We investigated the effects of vitamin D_3 on levels of activated MEK, a key mediator of signaling through the Ras-Raf1-MEK-Erk pathway, in SCC cells cultured in the presence or the absence of 12 % fetal bovine serum (FBS). Significant apoptosis (based on morphology) was observed under both culture conditions, but levels of apoptosis were greater under serum-starved conditions (data not shown). Immunoblot analysis using an anti-phospho-MEK antibody that selectively detects activated MEK (described in Experimental Procedures) was then performed on lysates prepared from attached and non-attached cells. As shown in Fig. 2, vitamin D_3 nearly completely inhibited MEK activation in the floating population of cells in the presence or the absence of serum, but had little effect on MEK phosphorylation in the attached populations from either vehicle- or vitamin D_3-treated cultures. Results from immunoblotting with an anti-MEK antibody demonstrated that the vitamin D_3-induced decrease in MEK phosphorylation was not simply due to inhibition of its activation but to down-regulation of MEK protein itself (Fig. 2). This result was confirmed using two other anti-MEK antibodies, which recognized distinct regions of the MEK protein (data not shown). Interestingly, the apoptotic, floating cells exhibited a lower molecular weight band that was also immunoreactive with the anti-MEK antibody (33 kDa in the presence of serum; 28 kDa in the absence of serum; Fig. 2A), suggesting that down-regulation of MEK protein levels might be due to MEK proteolysis.

Levels of phosphorylated/activated Erk1/2 were then analyzed and found to be virtually undetectable in the lysates of the floating cells (Fig. 2), indicating that the MEK-Erk signaling pathway is blocked in these cells. It is perhaps noteworthy that vitamin D_3 also modestly inhibited Erk1/2 in vitamin D_3-treated attached cells (Fig. 2) despite the fact that levels of phosphorylated MEK and intact MEK protein were unaffected (Fig. 2). The molecular basis of vitamin D_3-induced Erk1/2 inhibition in attached cells is currently under investigation. Erk1/2 expression was unaltered in lysates of vitamin D_3-treated attached cells, and, in contrast to MEK, was only slightly reduced in floating cells (Fig. 2). Reduction of Erk1/2 expression was more apparent under serum-starved conditions and was coincident with the appearance of a putative 23 kDa fragment (Fig. 2). Since culturing cells in the presence of serum is more physiologically relevant and since serum starvation appeared to promote activation of proteases that were not necessary for vitamin D_3-induced apoptosis or loss of MEK protein, all subsequent experiments were performed in the presence of 12 % FBS.

Since it is well established that programmed cell death often involves the activation of caspases, we examined whether these proteases might be responsible for vitamin D_3-induced MEK proteolysis. SCC cells were treated 2 days either with vehicle, vitamin D_3 alone, or vitamin D_3 in the presence of either DEVD-FMK or zVAD-FMK. Lysates were prepared and immunoblot analysis was carried out using anti-MEK antibody. While the caspase-3 inhibitor DEVD-FMK had little effect on vitamin D_3-induced MEK proteolysis, the pan-caspase inhibitor zVAD-FMK nearly completely blocked the loss of MEK protein and the production of the 33 kDa MEK fragment (Fig. 3). Thus, the decrease in MEK expression induced by vitamin D_3 treatment does not

appear to be due to inhibition of MEK protein synthesis, but rather the result of caspase-dependent MEK cleavage (although caspases may not cleave MEK directly). Caspase-dependent cleavage of MEK has not been previously reported. It is perhaps noteworthy that the apparent sum of the levels of intact MEK and the 33 kDa fragment in lysates of non-attached cells from vitamin D_3-cultures appears to be less than the total level of MEK in cells co-treated with vitamin D_3 and zVAD-FMK (Fig. 3, lanes 4 and 6). This suggests that the 33 kDa fragment is either less antigenic than intact MEK with the anti-MEK antibody used or that the 33 kDa fragment undergoes further proteolysis to yield products not recognized by the anti-MEK antibody or both. Finally, inhibition of MEK cleavage by zVAD-FMK resulted in the complete restoration of MEK and Erk1/2 phosphorylation/activation (Fig. 3), suggesting that MEK cleavage plays a critical role in the vitamin D_3-induced block of the MEK-Erk signaling pathway in detached cells.

Vitamin D_3 inhibits the Akt survival-signaling pathway. In addition to the MEK-Erk pathway, the PI-3-kinase-Akt pathway also generates a significant survival signal. Akt was recently demonstrated to undergo caspase-dependent cleavage during apoptosis induced by treatment either with etoposide, ultraviolet-C exposure, or Fas ligation in human Jurkat leukemia cells (52). To assess whether this pathway is also affected in vitamin D_3-induced apoptosis, immunoblot analysis was used to assess Akt phosphorylation and expression in lysates of cells treated with either vehicle or vitamin D_3. Figure 4A shows that vitamin D_3 affected Akt in a manner similar to that of MEK, inducing a strong decrease in both its phosphorylation and expression. Inhibition of Akt by vitamin D_3 was shown to occur via caspase-dependent cleavage of Akt (Fig. 4B).

Vitamin D_3 induces MEKK-1 expression in apoptotic and non-apoptotic cells and promotes MEKK-1 proteolysis in apoptotic cells. Although the above results clearly indicate that cells undergoing vitamin D_3-induced apoptosis exhibit a block in the mitogenic/survival signaling pathways, this alone may not be sufficient to induce cells to enter programmed cell death. Therefore, in an effort to assess whether vitamin D_3 treatment also activates stress signals that could directly promote apoptosis, SCC cells were treated with or without vitamin D_3 and the expression of MEKK-1, an upstream activator of the JNK and the p38 MAPK stress pathways, was assessed by Western blot analysis. As shown in Fig. 5A, vitamin D_3 significantly up-regulated MEKK-1 expression in both the attached and floating cell populations, with expression being greater in the floating, apoptotic cells. A second major immuno-reactive band of slightly lower molecular weight (approximately 190 kDa; Fig. 5A, left panel, lane 3) compared to that of intact MEKK-1 (MW = 196 kDa) was also detected in lysates of apoptotic, but not attached, cells. In addition, longer exposures of the X-ray film to the Western blot ECL signal revealed multiple minor immuno-reactive bands in the lysates of floating cells (MW range of 85 – 190 kDa) that were not observed in those of attached cells (Fig. 5A, right panel, compare lanes 2 and 3). Since the anti-MEKK-1 antibody recognizes an epitope lying very near the C-terminus of the MEKK-1 protein (within the last 50 amino acids of rat MEKK-1; see Experimental Procedures), these data indicate that the products formed represent MEKK-1 species that have undergone N-terminal proteolysis. It has been previously demonstrated that upon treatment of cells with either genotoxic agents or Fas ligation, MEKK-1 undergoes caspase-3-mediated cleavage, resulting in the removal of the N-terminal regulatory domain and the production of a constitutively active C-

terminal kinase domain (49). Thus, some or all of the lower molecular weight anti-MEKK-1 positive bands produced upon treatment with vitamin D_3 may represent MEKK-1 proteolytic products displaying constitutive kinase activity. Finally, as shown in Fig. 5B, MEKK-1 induction was observed by 22 h after treatment with vitamin D_3, a time at which vitamin D_3-induced apoptosis and cell detachment are not observed (data not shown), further suggesting a role for MEKK-1 in vitamin D_3–induced apoptosis. Induction of MEKK-1 by vitamin D_3 has not been previously reported.

MEK cleavage and MEKK-1 up-regulation are not significantly induced by cisplatin. We next addressed whether the effects of vitamin D_3 on MEK and MEKK-1 described above were general phenomena of SCC cells that could be observed during apoptosis induced by other agents or if they were selectively induced by treatment with vitamin D_3. Cells were treated either with vitamin D_3 (10 nM) or with cisplatin (cDDP, 1 μg/ml) for 2 days, the attached and non-attached cell populations were separated, and lysates were prepared from both. Similar levels of apoptosis were observed for vitamin D_3- and cisplatin-treated cells at the time of harvest (data not shown). As shown in Fig. 6, vitamin D_3 and cisplatin both induced PARP cleavage in the floating cell populations, but not in the attached cell populations, demonstrating that the floating cells from both treatments represent only those cells induced to undergo apoptosis. Significant loss of MEK expression and MEK cleavage, however, were only observed for lysates of apoptotic cells from vitamin D_3–treated cultures. Although the 33 kDa MEK fragment could be observed in lysates of cisplatin-treated floating cells, it was typically observed at levels less than those observed for lysates of vitamin D_3–treated floating cells (Fig. 6).

In addition, significant up-regulation of MEKK-1 was observed only for vitamin D_3–treated cells, particularly in the non-attached apoptotic population, and limited N-terminal MEKK-1 proteolysis was exclusively observed in the vitamin D_3–treated, detached cells (Fig. 6). Although a slight induction of MEKK-1 was observed for cisplatin-treated attached cells, this induction was no longer observed in the floating cells (Fig. 6). Thus, caspase-dependent MEK cleavage and MEKK-1 up-regulation/proteolysis are not general phenomena of apoptosis observed in these cells, but are selectively induced by vitamin D_3. Taken together, these results suggest that vitamin D_3 induces apoptosis in SCC cells by a mechanism that is distinct from that of genotoxic agents.

Vitamin D_3 and genotoxic agents display differential effects on the expression and activity of various anti-apoptotic and pro-apoptotic proteins. In an effort to more fully understand the molecular basis of vitamin D_3-induced apoptosis and how it compares with that of standard chemotherapeutic drugs, cells were treated with either vitamin D_3, cisplatin, or etoposide (VP16) for 2 days and the expression/phosphorylation of various members of the survival and stress signaling pathways were examined. Apoptosis in the floating cells was confirmed by assessing loss of intact PARP as an indicator of caspase activation (Fig. 7). In addition, actin expression was assessed to confirm equal loading of lysates to SDS-PAGE and to demonstrate selectivity of protein degradation. As shown in Figure 7, in the attached cells, phosphorylation of MEK and Erk1/2 was modestly inhibited by cisplatin and etoposide but was not affected by vitamin D_3. In the floating cells, the phosphorylation of these proteins was still moderately inhibited by the genotoxic agents, but was strongly decreased to virtually undetectable levels in lysates of

vitamin D_3-treated cells (Fig. 7). Moreover, cisplatin and etoposide had little effect on MEK expression in both the attached and the non-attached cell populations, whereas vitamin D_3 reduced MEK expression to nearly undetectable levels in non-attached cells (Fig. 7). Erk1/2 expression, while again being unaffected by cisplatin or etoposide, was only modestly reduced by vitamin D_3 in detached cells (Fig. 7). Expression of the pro-survival kinase Akt, on the other hand, was inhibited by all three agents in the non-attached cell populations, albeit to different extents (Fig. 7). Loss of Akt expression during apoptosis was expected since Akt was previously reported to undergo caspase-dependent cleavage in human Jurkat leukemia cells after treatment with various cytotoxic agents (52). It is interesting to note that a greater loss in Akt expression was exhibited by vitamin D_3 than by either cisplatin or etoposide, suggesting that vitamin D_3 induces greater activation of caspase(s) capable of cleaving Akt than either genotoxic agent. These results support the idea that inhibition of the MEK-Erk and Akt survival pathways may be a common mechanistic theme for agents that induce apoptosis (33,34,52). However, as might be expected, the molecular basis of inhibition of survival signals, as well as the extent to which they are inhibited, appears to vary among agents. Thus, the molecular mechanism by which vitamin D_3 and genotoxic agents inhibit Erk signaling appears to be distinct (i.e., MEK cleavage vs. decreased MEK phosphorylation, respectively), while these agents appear to down-regulate the Akt pathway in a similar manner (i.e., decreased Akt expression).

Divergent effects of vitamin D_3 and genotoxic drugs were also observed on the stress signaling proteins examined. First, similar to the results presented in Fig. 6, only vitamin D_3 significantly induced MEKK-1 expression, formation of the MEKK-1

doublet, and production of high molecular weight MEKK-1 proteolytic species (Fig. 7 and data not shown). Expression of SEK-1, a major downstream effector of MEKK-1, exhibited similar behavior to MEK, with strong down-regulation induced by vitamin D_3 in apoptotic cells but little effect exhibited by the DNA-damaging agents (Fig. 7). While genotoxic agents induced c-Jun phosphorylation, vitamin D_3 reduced levels of c-Jun phosphorylation (Fig. 7). Thus, vitamin D_3-induced apoptosis does not appear to be mediated via JNK-c-Jun activation. A second, distinct stress signaling pathway activated by MEKK-1, that mediated by p38 MAPK, appeared to remain intact after vitamin D_3 treatment, and demonstrated an increase in p38 MAPK phosphorylation levels in apoptotic cells, despite a modest decrease in p38 MAPK expression (Fig. 7). These data suggest that vitamin D_3-induced apoptosis is mediated by p38 MAPK. Unfortunately, we were unable to confirm the involvement of p38 MAPK through the use of the selective inhibitor SB203580 since this inhibitor also exhibited unexpected effects on some of the up-stream vitamin D_3-induced effects described above (data not shown). Further investigation is necessary to determine the exact role of p38 MAPK in vitamin D_3-induced apoptosis.

DISCUSSION

Taken together, the above results strongly suggest that vitamin D_3 induces programmed cell death in SCC cells via the induction of the pro-apoptotic signaling molecule MEKK-1 while blocking pro-survival signals from the MEK-Erk and Akt pathways. Based on these findings, we propose that, prior to commitment to apoptosis, vitamin D_3 up-regulates MEKK-1 in non-apoptotic, attached cells (Fig. 5, A and B), but at levels which are insufficient to overcome the opposing effects of the MEK-Erk and Akt survival pathways. Unknown factors then trigger limited activation of caspases, including an unidentified caspase (or caspase-dependent protease; discussed below) that is selectively activated in vitamin D_3-treated cells. This caspase activity promotes MEK cleavage and removal of the MEK-Erk pro-survival signal (Figs. 2 and 3). Furthermore, caspase-dependent proteolysis of Akt kinase occurs (52), and results in abrogation of this survival signal as well (Fig. 4). Finally, MEKK-1 undergoes partial proteolysis at its N-terminal regulatory domain (Figs. 5A and 6), producing species that exhibit constitutive activity, further activating caspases and significantly enhancing the pro-apoptotic signal (49). Activation of the MEKK-1-mediated stress pathway(s), without the presence of offsetting anti-apoptotic signals, is proposed to be sufficient for committing cells to enter apoptosis.

Caspase-dependent cleavage of MEK has not been previously reported. This may, in part, be explained by the fact that significant MEK cleavage was only observed for vitamin D_3–induced apoptosis and not for apoptosis induced by more commonly used cytotoxic agents (Figs. 6 and 7). Widmann et al. have recently performed an extensive

screening of more than thirty signaling proteins, including MEK, for their ability to undergo caspase-dependent proteolysis after induction of apoptosis by either treatment with etoposide, exposure to ultraviolet-C, or Fas ligation (52). They reported that, whereas all of the treatments induced apoptosis and the processing of caspase-3, as well as Akt cleavage, they had little effect on the expression of MEK (52). These results indicate that MEK cleavage is not a general phenomenon of the apoptotic program and suggest that a protease capable of cleaving MEK is selectively induced by vitamin D_3. It is important to note that while MEK cleavage is blocked by the pan-caspase inhibitor zVAD (Fig. 3), this does not necessarily indicate that caspases directly cleave MEK. The pro-apoptotic molecule Bax, for example, has been demonstrated to undergo caspase-dependent cleavage, but is not directly cleaved by caspases. Instead, caspase-3 first mediates the cleavage and consequent activation of calpain, which then acts to cleave Bax (53,54). Identification of the caspase(s)/protease(s) responsible for MEK cleavage is an area of current research.

It is somewhat surprising that vitamin D_3 would also strongly down-regulate (in caspase-dependent fashion; data not shown) the stress signaling molecule SEK-1 in apoptotic cells, similar to MEK (Fig. 7). However, other models have shown a lack of correlation to exist between activation of the SEK-JNK pathway and induction of apoptosis (55,56). In fact, for some cells, signaling in this pathway has been linked to cell survival (57) and even to cell proliferation (58). Thus, the possibility exists that elimination of SEK-1 signaling may actually assist in commitment to the cell death program. As with MEK cleavage, caspase-dependent SEK-1 proteolysis has not been previously described. It is interesting to note that MEK and SEK-1 have analogous

central positions in their respective three-kinase signaling modules (reviewed in (59)) and that both should be efficiently targeted for destruction in a vitamin D_3-selective fashion. Thus, the possibility exists that a single, vitamin D_3-induced, caspase/protease activity is capable of cleaving these critical signaling molecules and irreversibly blocking their respective pathways.

As mentioned above, Akt, in contrast to MEK and SEK-1, appears to be down-regulated in vitamin D_3-treated apoptotic cells as part of the general apoptotic program since it was also observed after treatment with genotoxic agents (Fig. 7). Co-treatment with zVAD blocked vitamin D_3-induced loss of Akt expression, indicating that it is also caspase-dependent (Fig. 4B). These findings support previous work which demonstrate caspase-dependent cleavage of Akt upon induction of apoptosis by DNA-damaging agents or Fas ligation (52). It is presently not known whether vitamin D_3-induced Akt down-regulation is mediated by the same caspase(s) activated by genotoxic agents or if distinct activities may be involved.

MEKK-1 has been demonstrated to play a pivotal role in mediating apoptosis induced by various cytotoxic treatments including genotoxins (49) and Fas ligation (50). MEKK-1 is known to be activated by phosphorylation [autophosphorylation or via a distinct kinase (60,61)] and/or caspase-3-mediated cleavage (49). Treatment of cells with DNA-damaging agents results initially in phosphorylation-dependent activation of MEKK-1 and, subsequently, in its caspase-3-mediated cleavage, leading to nearly complete loss of the 196 kDa intact protein and the appearance of a 91 kDa fragment with constitutive kinase activity (49,52). Interestingly, this pattern is not maintained in cells induced to undergo apoptosis by vitamin D_3. Instead, intact MEKK-1 expression is

further induced in apoptotic cells concurrent with the expression of a slightly lower molecular weight MEKK-1 species (about 190 kDa) as well as multiple smaller MEKK-1 fragments representing N-terminal proteolytic species (Fig. 5). The cellular scaffolding protein 14-3-3 has been shown to associate with the N-terminal portion of MEKK-1 and is thought to regulate its biological function by sequestering the protein to membranes, enabling it to respond to growth factor and cytokine stimulation (62). Whereas endogenous MEKK-1 has been shown to be exclusively associated with membranes, MEKK-1 over-expression leads to significant accumulation of the protein within the cytosol (about half of the expressed protein), uncontrolled MEKK-1 activation, and cell death (49). Similarly, caspase-3-mediated cleavage not only renders the C-terminal kinase domain to be constitutively active but also liberates it from membranes (50,62). Thus, deregulated localization of MEKK-1 within the cell appears to play a role in the induction of apoptosis via MEKK-1. At present, it is not known whether the intact MEKK-1 protein and/or the N-terminal proteolytic fragments induced by vitamin D_3 are still associated with 14-3-3 or whether they are expressed in the cytosol.

The results presented herein shed light on the molecular events involved in vitamin D_3–induced apoptosis and provide a biochemical basis for the use of vitamin D_3 (and vitamin D_3 analogues) in the treatment of cancer. Indeed, clinical trials are currently underway to assess the efficacy of vitamin D-based compounds both as a single agent (63,64) as well as in combination with traditional chemotherapeutic agents (65). Several studies have demonstrated that vitamin D_3 or its analogues can enhance the in vitro cytotoxicity of various anti-neoplastic agents, including tumor necrosis factor α (TNF-α) (66), radiation (67), cisplatin (51,68,69), adriamycin (69,70), and paclitaxel (70). In all

but the earliest of these studies (68), enhancement of cytotoxicity required pre-treatment of the cells with vitamin D_3. It is intriguing to speculate, based on the results described above, that vitamin D_3 may be priming the cells to exhibit heightened sensitivity to the cytotoxic regimen by either up-regulating MEKK-1 expression/activity, inducing a vitamin D_3-selective caspase/protease, or both. This notion gains support from studies demonstrating that acute expression of activated MEKK-1 potentiates apoptosis induced by low doses of DNA-damaging agents or TNF-α (71). Moreover, MCF-7 cells, which do not express caspase-3 but require its expression for cisplatin-induced apoptosis (72), show enhanced cisplatin cytotoxicity when pre-treated with vitamin D_3 (68), suggesting that vitamin D_3 may selectively induce a caspase-3-like activity in these cells that renders them susceptible to cisplatin. In a similar fashion, vitamin D_3 may alleviate drug resistance that is based on caspase-3 deficiency as in cisplatin-resistant human ovarian adenocarcinoma 2008/C13 cells (73). Furthermore, vitamin D_3 may attenuate drug resistance that is based on the MEK-Erk (reviewed in (34)) or Akt (74) survival pathways, via induction of a caspase/protease activity that interferes with these signaling mechanisms.

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FOOTNOTES

[1]The abbreviations used are: vitamin D_3, 1,25-dihydroxycholecalciferol; SCC, squamous cell carcinoma; Erk, extracellular signal-regulated protein kinase; MEK, Erk kinase; MEKK-1, mitogen-activated protein kinase kinase kinase-1; FBS, fetal bovine serum; PARP, poly(ADP-ribose) polymerase; ECL, enhanced chemiluminescence; TNF-α, tumor necrosis factor-α.

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FIGURE LEGENDS

FIGURE 1. Vitamin D_3 induces apoptosis in murine squamous cell carcinoma (SCC) cells in vitro. SCC cells were treated with either vehicle (< 0.001 % ethanol, EtOH) or 10 nM vitamin D_3 (1,25-D3) in RPMI medium containing 12 % fetal bovine serum (FBS), as described in *Experimental Procedures*. After 2 days, cells were photographed (*A*) and/or processed for either YO-PRO-1/propidium iodide staining followed by flow cytometric analysis (*B*) or Western blotting (*C*) as described in *Experimental Procedures*. Photographs show that vitamin D_3 causes SCC cells both to flatten (G1 arrest) and to enter apoptosis as visualized by bright cells that exhibit cellular condensation and membrane blebbing. Results from flow cytometric analysis were plotted such that FL1 represents fluorescence of propidium iodide and FL2 represents the fluorescence of YO-PRO-1. This analysis demonstrates that vitamin D_3 significantly increases the percent of cells in early apoptosis (from 0.2 % to 11.0 %), cells in late apoptosis/necrosis (from 13.7 % to 28.7 %), and total cells in apoptosis (from 13.9 % to 39.7 %). Immunoblot analysis with anti-PARP antibody indicates that cells which are detached (but not the attached cells) are apoptotic. ❊ In vehicle-treated cultures, nearly all of the cells were attached to the flask and it was not possible to collect the very small number of floating cells for Western blot analysis. These results are representative of at least four independent experiments.

FIGURE 2. Vitamin D_3 induces loss of MEK protein expression, appearance of lower molecular weight anti-MEK immunoreactive proteins, and strong inhibition of MEK-Erk1/2 signaling. Cells were treated 2 d with either vehicle or 10 nM vitamin D_3 in the presence or the absence of fetal bovine serum (FBS). The floating and attached cell populations were then obtained and subsequently processed for immunoblotting with either anti-phospho-MEK, anti-phospho-Erk1/2, anti-MEK, or anti-Erk1/2 antibodies to assess the phosphorylation/activation and expression of MEK and Erk1/2, as described in *Experimental Procedures*. Immunoblot analysis with anti-MEK antibody detected putative MEK fragments at 33 kDa (in the presence of serum) and 28 kDa (in the absence of serum) in apoptotic cell populations from vitamin D_3-treated cultures. ❊ Vehicle-treated cells were essentially all attached to the flasks whether or not serum was present. These results are representative of at least three independent experiments.

FIGURE 3. Vitamin D_3 induces caspase-dependent MEK cleavage. Cells were treated 2 d with either vehicle or 10 nM vitamin D_3 in the presence or the absence of either 20 μM Z-DEVD-FMK (DEVD) or 20 μM Z-VAD(OMe)-FMK (zVAD). Cells were then harvested and lysates were prepared and subjected to immunoblotting with appropriate antibodies, as described in *Experimental Procedures*. The results presented in the figure were obtained from a single X-ray exposure of samples run on the same gel using equivalent amounts of protein for each sample. ❊ Vehicle-treated cells were essentially all attached to the flask. Inhibition of MEK cleavage by zVAD was observed in at least three independent experiments.

FIGURE 4. Vitamin D_3 inhibits Akt survival signaling via caspase-dependent Akt cleavage. Cells were treated 2 d with either vehicle or 10 nM vitamin D_3 (*A* and *B*) in the presence or the absence of 20 μM Z-VAD(OMe)-FMK (zVAD) (*B*). Cells were then processed for immunoblotting with either anti-phospho-Akt or anti-Akt antibodies, as described in *Experimental Procedures*. It should be noted that, in some experiments, vitamin D_3 partially inhibited phosphorylation of Akt in the attached cells.

FIGURE 5. Vitamin D_3 induces MEKK-1 expression and N-terminal proteolysis in apoptotic cells. *A*, cells were treated 2 d and then processed for anti-MEKK-1 immunoblotting to assess MEKK-1 expression as described in *Experimental Procedures*. The anti-MEKK-1 antibody recognizes an epitope located within the last 50 amino acids of the rat MEKK-1 protein and is identical in sequence to the same region of the mouse MEKK-1 protein (see *Experimental Procedures*). *B*, cells were treated for either 1 or 2 d and then processed as in *A*. Vitamin D_3-induced MEKK-1 up-regulation and N-terminal proteolysis were observed in at least four independent experiments.

FIGURE 6. Vitamin D_3 and cisplatin both induce PARP cleavage but only vitamin D_3 induces significant down-regulation of MEK and up-regulation/N-terminal proteolysis of MEKK-1. Cells were treated 2 d with either vehicle, 10 nM vitamin D_3 or 1 μg/ml cisplatin (cDDP) and processed for immunoblotting to assess PARP cleavage, MEK expression/cleavage, and MEKK-1 expression/proteolysis as described in *Experimental Procedures*. These results suggest that MEK cleavage and MEKK-1 up-regulation are not general phenomena of apoptosis (as is PARP cleavage), but are selectively induced by vitamin D_3. These results are representative of four independent experiments.

FIGURE 7. Vitamin D_3 and genotoxic agents exhibit similar and divergent effects on the expression and phosphorylation of various signaling proteins. SCC cells were treated 2 d with either vehicle, 10 nM vitamin D_3, 10 μM etoposide (VP16), or 1 μg/ml cisplatin (cDDP), harvested, and lysates prepared as described in *Experimental Procedures*. Immunoblotting was then carried out to assess the expression of PARP, MEK, Erk1/2, Akt, MEKK-1, SEK-1, p38 MAPK, and actin, as well as the phosphorylation/activity of MEK, Erk1/2, c-Jun, and p38 MAPK, as described in *Experimental Procedures*. These results suggest that the molecular mechanism by which vitamin D_3 promotes apoptosis is distinct from that of genotoxic drugs. It should be noted that, although genotoxic drugs consistently increased (or maintained) levels of phosphorylation for p38 MAPK and c-Jun in the attached cell population, as shown here, their affects on the levels of these phospho-proteins in the floating cell population were variable.

Fig. 1

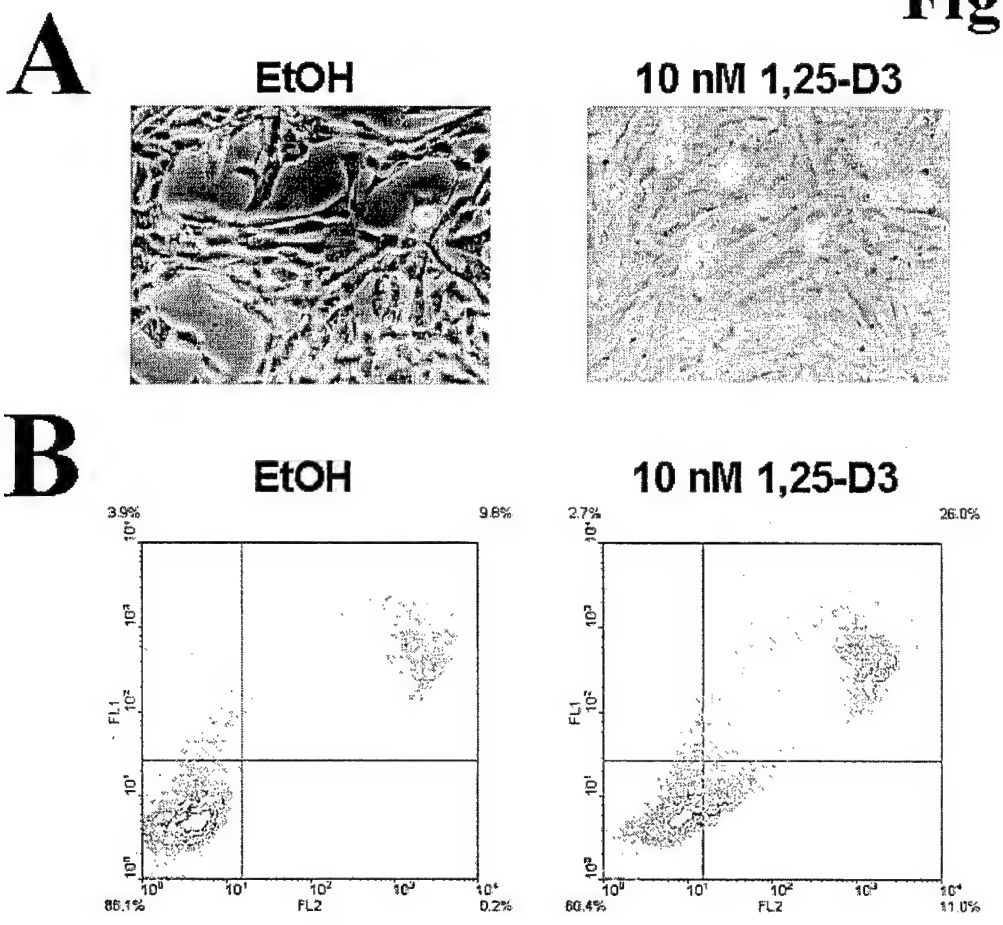

A
EtOH
10 nM 1,25-D3
B
EtOH
10 nM 1,25-D3
3.9%
9.8%
86.1%
0.2%
2.7%
26.0%
60.4%
11.0%
FL1
FL2
C
EtOH
1,25-D3, Attached
1,25-D3, Floating
PARP
85 kDa Frag.

Fig. 2

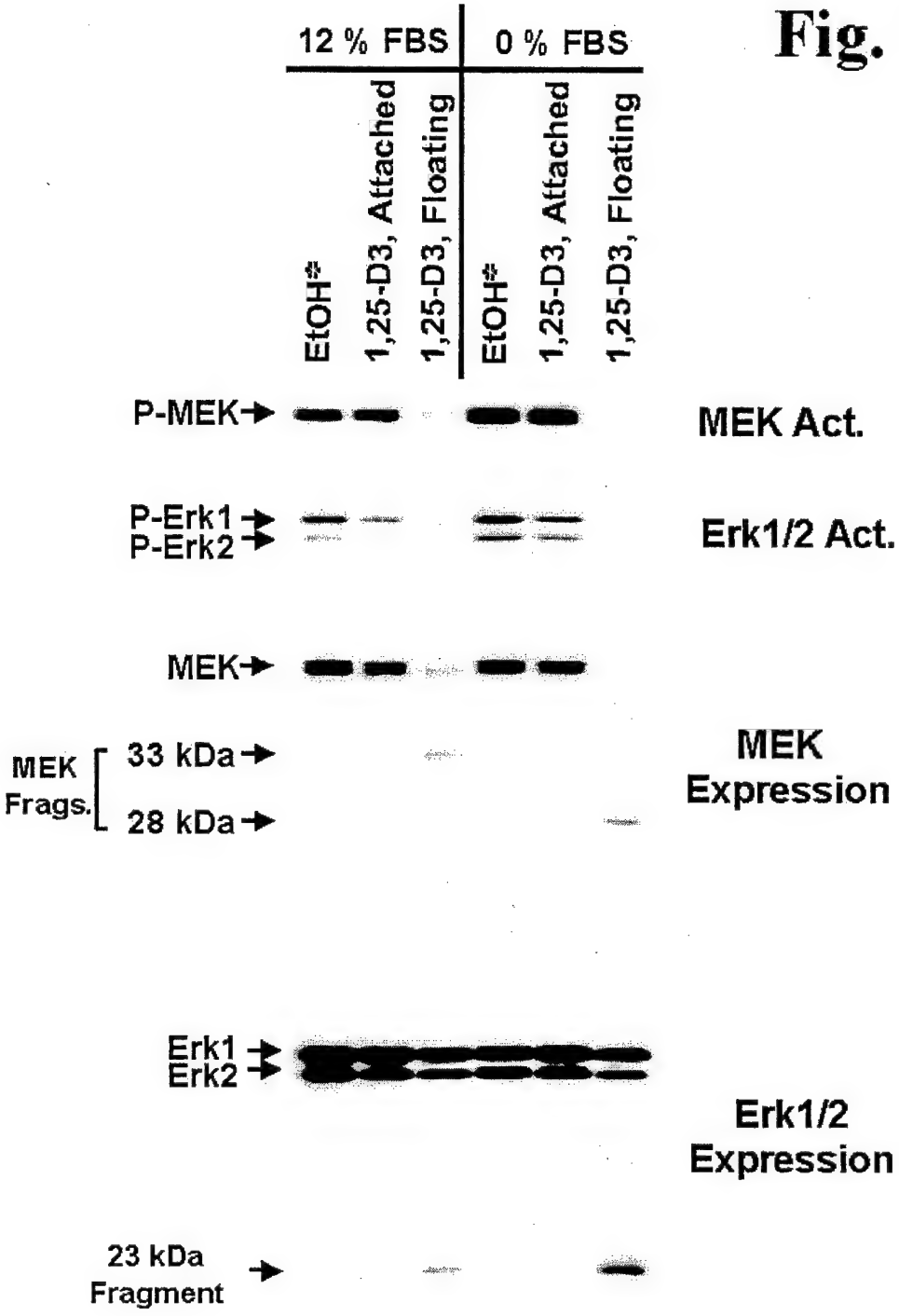

12 % FBS
0 % FBS
EtOH
1,25-D3, Attached
1,25-D3, Floating
EtOH
1,25-D3, Attached
1,25-D3, Floating
P-MEK
MEK Act.
P-Erk1
P-Erk2
Erk1/2 Act.
MEK
MEK Frags.
33 kDa
28 kDa
MEK Expression
Erk1
Erk2
Erk1/2 Expression
23 kDa Fragment

Fig. 3

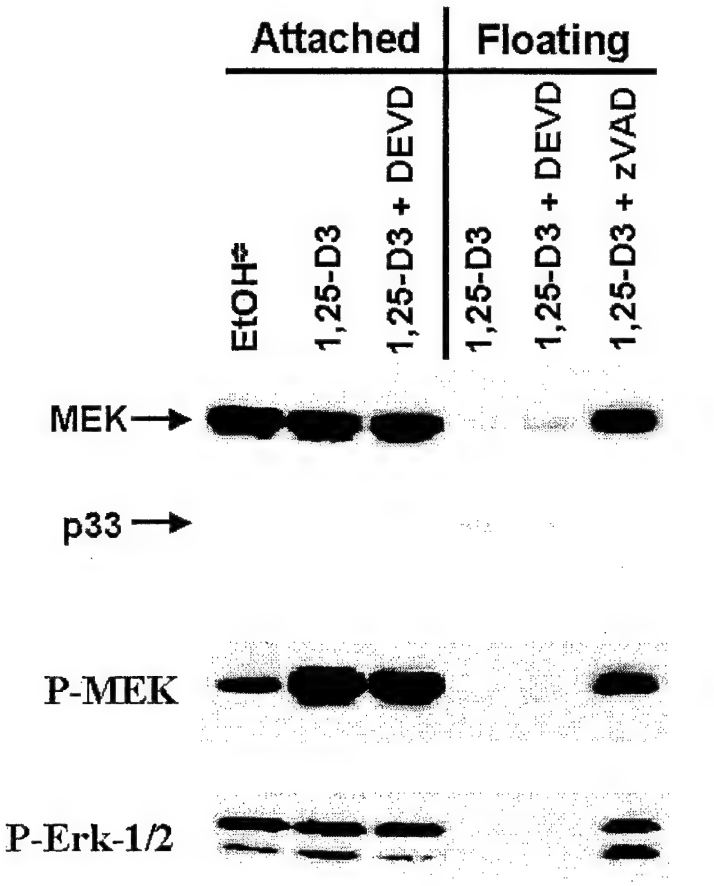
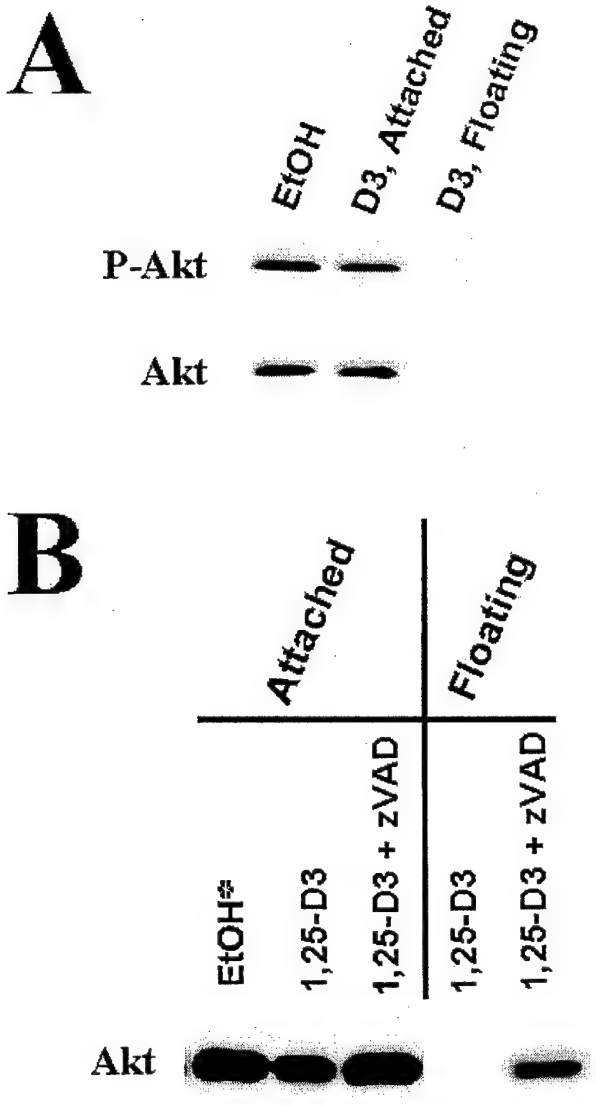

Attached
Floating
EtOH
1,25-D3
1,25-D3 + DEVD
1,25-D3
1,25-D3 + DEVD
1,25-D3 + zVAD
MEK
p33
P-MEK
P-Erk-1/2

Fig. 4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D3, Attached
D3, Floating
P-Akt
Akt
B
Attached
Floating
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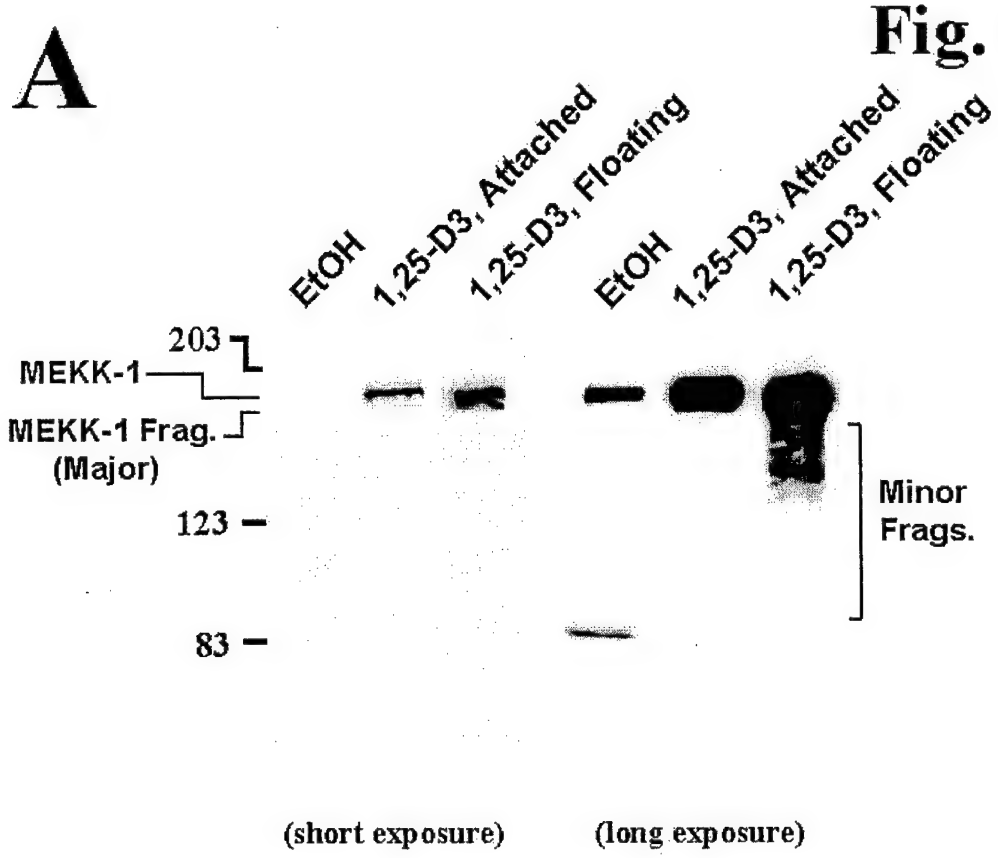
1,25-D3
1,25-D3 + zVAD
1,25-D3
1,25-D3 + zVAD
Akt

Fig. 5


A
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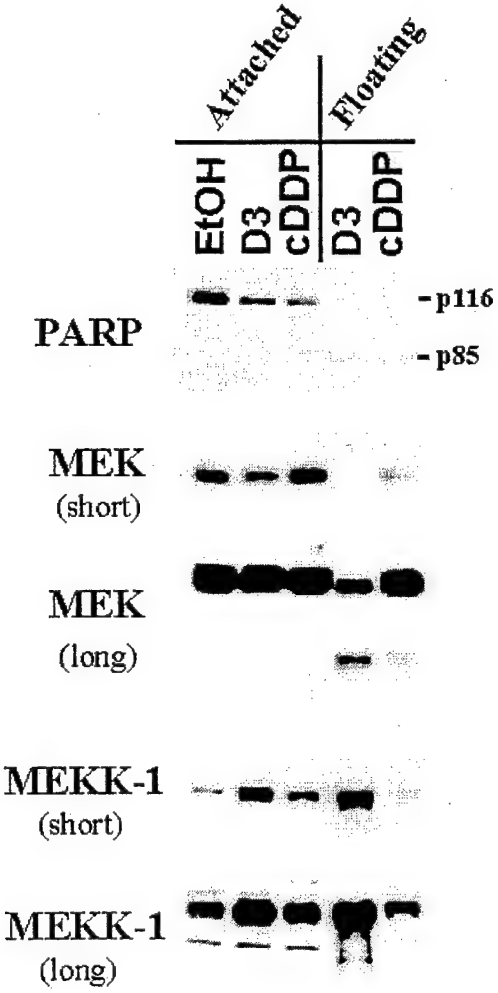
1,25-D3, Floating
EtOH
1,25-D3, Attached
1,25-D3, Floating
203
MEKK-1
MEKK-1 Frag.
(Major)
123
83
Minor
Frags.
(short exposure)
(long exposure)
B
EtOH
1,25-D3, Attached
EtOH
1,25-D3, Attached
1,25-D3, Floating
MEKK-1
22 h
45 h

Fig. 6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Floating
EtOH
D3
cDDP
D3
cDDP
PARP
- p116
- p85
MEK
(short)
MEK
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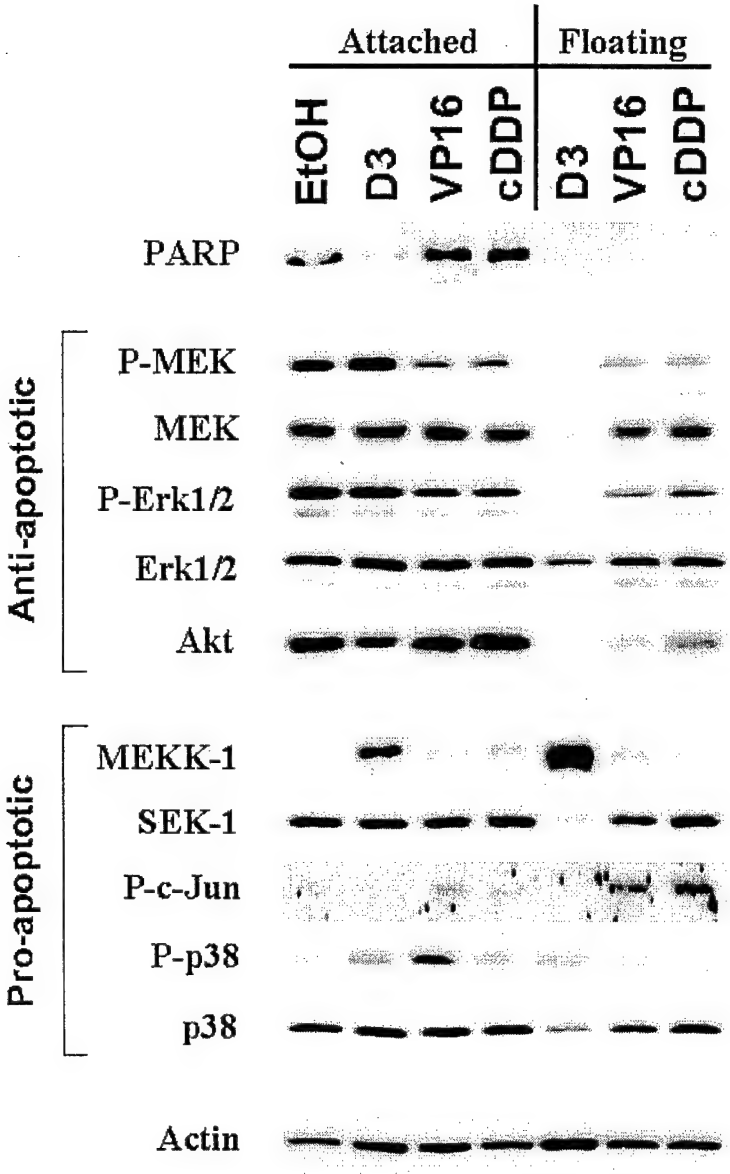
MEKK-1
(short)
MEKK-1
(long)

Fig. 7


Attached
Floating
EtOH
D3
VP16
cDDP
D3
VP16
cDDP
PARP
Anti-apoptotic
P-MEK
MEK
P-Erk1/2
Erk1/2
Akt
Pro-apoptotic
MEKK-1
SEK-1
P-c-Jun
P-p38
p38
Actin

*820

Effects of High Dose Calcitriol (1,25 Dihydroxyvitamin D3) on the Pharmacokinetics of Paclitaxel or Carboplatin: Results of Two Phase I Studies. *C. S. Johnson, M. J. Egorin, E. Zuhowski, R. Parise, M. Cappozolli, C. P. Belani, G. S. Long, J. Muindi, D. L. Trump; Univ of Pittsburgh Cancer Inst, Pittsburgh, PA*

Considerable in vitro and in vivo data indicate that pretreatment with calcitriol enhances the antitumor effects of taxanes and platinum analogues. We are conducting two phase I trials: carboplatin (AUC=5) q28 days + escalating doses of calcitriol (subcutaneous) QDx3 q28 days and paclitaxel (80mg/m^2) weekly + escalating doses of calcitriol (p.o.) QDx3, weekly. Calcitriol starting dose was 4μ g QDX3 in each trial. Studies are designed such that in each patient, carboplatin or paclitaxel are given on day 1 before calcitriol in one treatment and on day 3 after two days of high dose calcitriol in the next. This permits comparison of AUC of either agent in the same patient before and after pretreatment with calcitriol. Dose-limiting toxicity has not been encountered in either trial; current doses of calcitriol are 13μg po QDX3 either weekly (with paclitaxel) or q4 week (with carboplatin). AUC of paclitaxel was not different with or without calcitriol. AUC of carboplatin was higher in each patient following calcitriol than before calcitriol (mean AUC = 7.8μ g/ml.hr $\pm$ 1.3, carboplatin D1 vs. AUC = 6.7μ g/ml.hr $\pm$ 1.5, carboplatin day 3). While no dose limiting toxicity has been seen, myelosuppression following the sequence carboplatin$\rightarrow$ calcitriol was consistently less than that following calcitriol $\rightarrow$ carboplatin, consistent with the change in AUC. No clinically detectable renal impairment has been seen with either sequence. These data indicate that potentiation of paclitaxel by calcitriol is not related to altered pharmacokinetics of paclitaxel while potentiation of carboplatin by calcitriol may in part be related to reduced carboplatin clearance. Studies to characterize these effects further are underway. Supported by NIH/NCRR/GCRC Grant #5M01RR00056 and NCI CA67267, CaPCURE and Bristol-Meyers-Squibb.

*1327

High Dose Calcitriol ($1,25(OH)_2$ Vitamin D_3) + Dexamethasone in Androgen Independent Prostate Cancer (AIPC). *D. L. Trump, S. Serafine, A. Brufsky, J. Muindi, R. Bernardi, D. Potter, C. Johnson; Univ of Pittsburgh Cancer Inst, Pittsburgh, PA*

Epidemiological data suggest that low vitamin D exposure increases the risk of prostate cancer and in vitro and in vivo data demonstrate the antitumor effects of calcitriol in prostate cancer models. We are conducting a phase II trial of high dose calcitriol + dexamethasone in AIPC. We have shown that dexamethasone (Dex) enhances calcitriol antitumor effects and reduces calcitriol-induced hypercalcemia. Thirty-two (32) patients with AIPC progressing despite anti-androgen withdrawal have been entered. Calcitriol is given orally, 8μg Monday, Tuesday, Wednesday (MTW), weekly x1 month, 10μg MTW, weekly x1 month and then 12μg MTW weekly. Dex is given 4 mg Sunday, MTW each week. Patients are evaluable for response if they completed $\geq$ 1 month of 12 μg calcitriol MTW. 24 patients are evaluable, 4 TETE 4 unevaluable. 2 patients have had mild and transient hypercalcemia. (12.1 mg/dL in each case). In neither case was cessation of treatment or dose reduction required. 3 patients withdrew from therapy due to moderate hypercortisolism (proximal myopathy) and one patient developed an asymptomatic renal stone. Among 24 patients evaluable, responses have been noted as follows: > 50% PSA $\downarrow$: 5 (21%), $\downarrow$ PSA velocity: 19 (79%). These data indicate that high dose calcitriol + dexamethasone is safe; clear antitumor effects are evident. Studies continue to define the maximum dose of calcitriol which can be safely administered on this intermittent schedule and to evaluate the mechanisms of calcitriol effects.
Supported by CaPCURE, NCI CA 47904 and NCI CA 67267

#97 ENHANCED ANTI-TUMOR EFFICACY WITH DEXAMETHASONE/CALCITRIOL/CISPLATIN THERAPY: ROLE OF $P21^{WAF1}$. Pamela A Hershberger, R. A Modzelewski, R. M Reuger, K. E Blum, D. L Trump, and C. S Johnson, *Univ of Pittsburgh Cancer Institute, Pittsburgh, PA*

Calcitriol ($1,25\text{-}D_3$) pre-treatment decreases p21 expression and increases cisplatin (cDDP) cytotoxicity in the murine squamous cell carcinoma (SCC) model. Dexamethasone (DEX) also decreases p21 expression in SCC *in vitro* and was evaluated for its ability to increase cDDP cytotoxicity in combination with $1,25\text{-}D_3$. SCC cells were incubated with DEX, $1,25\text{-}D_3$, and/or cDDP, and cell viability determined by trypan blue. Pre-treatment with DEX/$1;25\text{-}D_3$ followed by cDDP resulted in greater growth inhibition than treatment with cDDP alone or pre-treatment with $1,25\text{-}D_3$ followed by cDDP. To examine the *in vivo* activity of the combination, SCC tumor-bearing mice were treated with DEX on days 0 to 3, $1,25\text{-}D_3$ on days 1 to 3, and cDDP on day 3. Greater anti-proliferative activity was observed for DEX/$1,25\text{-}D_3$/cDDP as compared to DEX/$1,25\text{-}D_3$($p<0.003$, Mann-Whitney test) or $1,25\text{-}D_3$/cDDP ($p<0.05$). Preliminary Western analysis of tumors harvested from treated animals indicates p21 protein levels are increased following DEX/$1,25\text{-}D_3$/cDDP administration compared to $1,25\text{-}D_3$/cDDP. These observations suggest DEX enhancement of anti-tumor activity is independent of p21. Nonetheless, the observed increase in anti-tumor activity supports the hypothesis that DEX/$1,25\text{-}D_3$/cDDP therapy has utility in the treatment of solid tumors. Supported by NIH grant CA67267, CaPCURE, The M.H. Jennings Fnd., and USAMRMC.

#1787 THE BISPHOSPHONATE ZOLEDRONATE (CGP42446) SIGNIFICANTLY DECREASES CALCITRIOL MEDIATED HYPERCALCEMIA. Robert M Rueger, K. E Blum, C. S Johnson, and D. L Trump, *Univ of Pittsburgh Cancer Institute, Pittsburgh, PA*

Bisphosphonates are used to inhibit increased bone resorption in tumor-induced hypercalcemia, Paget disease, and osteoporosis. The bisphosphonates are calcium chelators and have high affinity for bone. Zoledronate, a new bisphosphonate, has substantially increased antiresorptive potency compared to first generation agents (e.g. etidronate). Recent studies indicate that Zoledronate may block calcitriol-induced osteolysis in vitro. Detailed studies were initiated to examine the effect of Zoledronate on calcitriol-induced hypercalcemia. Normal C3H/HeJ mice were pretreated with Zoledronate (10μg/kg) on day-1 and given daily x3 calcitriol (0.251μg/mouse). Blood was collected 0, 24, 48 hours following the last calcitriol treatment. Peak serum calcium levels were significantly decreased in Zoledronate/calcitriol treated animals as compared to calcitriol alone ($p=0.0002$). 24 and 48 hours after the third calcitriol treatment, serum calcium was elevated in animals treated with calcitriol alone (17.2 ± 1.1 and 16.5 ± 1.1mg/dl, respectively) but significantly reduced in animals treated with the combination (14.7 ± 0.9 and 13.4 ± 0.9mg/dl). In addition, Zoledronate alone and Zoledronate/calcitriol treated animals showed a marked reduction in dehydration, piloerection, and cachexia due to hypercalcemia. These data indicate that Zoledronate blocks the only known calcitriol toxicity and suggest that Zoledronate may permit administration of high doses of calcitriol. Supported by NIH grant CA 67267, CaPCURE, USAMRMC, Novartis Pharm. and The Mary Hillman Jennings Foundation.

#2724 1,25-DIHYDROXYCHOLECALCIFEROL (CALCITRIOL) INHIBITS MITOGEN-ACTIVATED PROTEIN KINASE ACTIVITY WITHOUT SIGNIFICANTLY AFFECTING MEK ACTIVITY IN MURINE SQUAMOUS CELL CARCINOMA CELLS. Terence F McGuire, J. Brailler, D. L Trump, and C. S Johnson, *Univ of Pittsburgh Cancer Institute, Pittsburgh, PA*

We demonstrated that calcitriol inhibits the growth of tumor cells in vitro and in vivo in a variety of tumor models. The mechanism by which calcitriol exerts its growth inhibitory effects is unclear. In vitro, exponentially growing murine squamous cell carcinoma (SCC) cells express high levels of phosphorylated/activated MAPK (erk1 and erk2), proteins which are known to transduce mitogenic signals to the nucleus in response to a number of extracellular stimuli. Treatment of cells with calcitriol (10 nM) decreased levels of phosphorylated (activated) MAPK at 24 h and 48 h with no significant effect on MAPK expression as evaluated by Western blot. While calcitriol modestly reduced the expression of MEK, the kinase responsible for activating MAPK, it did not significantly alter MEK phosphorylation/activity as assessed by Western blot and in vitro kinase assays. In addition, the levels of EGF, PDGF, and IGF1 growth factor receptors were significantly enhanced in calcitriol-treated cells compared to vehicle-treated cells. Thus, increased growth factor receptor signaling may compensate for the loss in MEK expression induced by calcitriol. These results suggest that calcitriol does not inhibit MAPK by inhibiting the upstream mitogenic signal from growth factor receptors but rather may be deactivating MAPK by inducing a MAPK phosphatase, MKP-1, which appears to be modulated. Supported by grants from NIH CA67267, CaPCURE, USAMRMC, and The M. H. Jennings Fnd.

#4908 EFFECTS OF CALCITRIOL ON THE GLUCOCORTICOID RECEPTOR AND THE ROLE OF CROSS-TALK IN THE ANTI-PROLIFERATIVE EFFECTS OF THE COMBINATION OF CALCITRIOL AND DEXAMETHASONE.

Wei-Dong Yu, R. J Bernardi, P. A Hershberger, C. S Johnson, and D. L Trump, *Univ of Pittsburgh Cancer Institute, Pittsburgh, PA*

We have demonstrated that the anti-proliferative effects of calcitriol (1,25-D_3) are significantly enhanced by dexamethasone (DEX) *in vitro* and *in vivo* using the SCCVII/SF squamous cell carcinoma model system. DEX increases vitamin D receptor (VDR) ligand binding and VDR protein levels. Studies were undertaken to assess the interaction between 1,25-D_3, DEX, and the glucocorticoid receptor (GR), and its importance for the anti-proliferative effects of this combination therapy. We used the GR antagonist, RU486, to assess the requirement for transcriptional regulation by DEX. Using a whole cell ligand binding assay, we demonstrate that GR ligand binding was significantly increased by treatment of SCC cells with 1,25-D_3 ($P<0.001$), while binding was decreased in cells treated with 1,25-D_3 and RU486. Western blot analysis revealed that treatment of SCC cells with 1,25-D_3 increased GR protein levels as compared to untreated cells. RU486 blocked the growth inhibitory effects of DEX *in vitro* by crystal violet assay and blocked accumulation of cells in G_0/G_1. Our results are consistent with a model in which GR binding is required for the enhancement of the anti-proliferative effects of 1,25-D_3 by DEX. Furthermore, they indicate that cross-talk between 1,25-D_3 and DEX signaling may be an important part of the mechanism of action of this combination therapy. Supported by NIH grant CA 67267, CaPCURE, USAMRMC, and The Mary Hillman Jennings Foundation.

#454 1,25-Dihydroxycholecalciferol (Calcitriol) Enhancement of Chemotherapeutic Efficacy: Synergistic Effects by Median Dose Effect. Wei-Dong Yu, Robert M. Rueger, Ralph W. Fuller, Candace S. Johnson, and Donald L. Trump. *University of Pittsburgh Cancer Institute, Pittsburgh, PA.*

1,25-dihyroxycholecalciferol (calcitriol) or the calcitriol analogue, 1,25-dihydroxy-16-ene-23-yne-cholecalciferol, 7553 (Ilex) significantly increases in vitro and in vivo mediated anti-tumor effects of platinum analogues (cisplatin and carboplatin) and taxanes (paclitaxel and docetaxel) in a variety of tumor model systems (murine syngeneic squamous cell carcinoma, SCC; human xenograft prostatic adenocarcinoma, PC-3 and lung carcinoma, MV522 and a rat syngeneic prostatic adenocarcinoma, MLL). The effect of these combinations results in greater than 3-5 log cell kill when compared to any agent alone. To determine whether the ability to enhance calcitriol-mediated antitumor effect was limited to these class of drugs, we investigated the ability of calcitriol to potentiate the cytotoxic activity of irinotecan (CPT-11), etoposide (VP-16), mitoxantrone (MXN), 5-fluorouacil (5FU), carmustine (BCNU), cytarabine (Ara-C), doxorubincin (DOX) in SCC by the in vitro crystal violet/MTT assay. Using median dose effect analysis, strong synergy was observed across all fractions effected with CPT-11, Ara-C, DOX, 5FU, and MXN with no significant enhancement seen with BCNU and moderate synergy with VP-16. In synergistic combinations, lower doses of both calcitriol and chemotherapeutic drug were equally effective suggesting the potential to significantly limit toxicity. These results demonstrate differences in the ability of calcitriol to increase the antitumor efficacy of cytotoxic agents. The mechanisms involved in the ability of calcitriol to significantly enhance these agents remains to be determined and is currently under investigation. Supported by NIH grant CA 67267; CaPCURE, Ilex Oncology, Inc., and USAMRMC 17-98-1-8549.

#3449 1,25-Dihydroxyvitamin D3 (Calcitriol) Inhibits Survival Signals and Induces MEKK-1 and Apoptosis in Murine Squamous Cell Carcinoma Cells: Selective Induction of Caspase-dependent MEK Cleavage. Terence F. McGuire, Donald L. Trump, and Candace S. Johnson. *University of Pittsburgh, Pittsburgh, PA.*

Calcitriol inhibits proliferation and induces apoptosis in several human cancer lines in vitro and in vivo. However, little is known about the molecular events involved in calcitriol-induced apoptosis. Here, we demonstrate that the growth-promoting/pro-survival signaling molecule mitogen-activated protein kinase kinase (MEK) is cleaved in a caspase-dependent manner in murine squamous cell carcinoma (SCC) cells induced to undergo apoptosis by treatment with calcitriol. MEK cleavage has not been previously reported. Cleavage results in nearly complete loss of intact MEK expression and, consequently, of MEK and Erk1/2 phosphorylation. Erk1/2 expression is only slightly affected. Similar to MEK, the phosphorylation/expression of Akt, a kinase regulating a second cell survival pathway, is also inhibited after calcitriol treatment. In contrast, the pro-apoptotic signaling molecule MEKK-1 is up-regulated in both apoptotic and non-apoptotic cells with greater induction and partial N-terminal proteolysis of MEKK-1 observed in apoptotic cells. In contrast to calcitriol, cisplatin and etoposide do not promote significant loss of MEK expression, and do not up-regulate MEKK-1. We propose that calcitriol induces apoptosis in SCC cells by a mechanism involving selective induction of MEKK-1 and promotion of caspase-dependent MEK cleavage. These effects, together with more general features of programmed cell death (i.e., caspase-dependent cleavage of Akt), shift the overall state of the cell to one strongly favoring apoptosis. Supported by grants from NCI CA85142, CA67267, USAMRC 179818549, and CaPCURE.